

Perturbations of *Drosophila* α -Actinin Cause Muscle Paralysis, Weakness, and Atrophy but Do Not Confer Obvious Nonmuscle Phenotypes

Erica M. Roulier, Christine Fyrberg, Eric Fyrberg

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Abstract. We have investigated accumulation of α -actinin, the principal cross-linker of actin filaments, in four *Drosophila fliA* mutants. A single gene is variably spliced to generate one nonmuscle and two muscle isoforms whose primary sequence differences are confined to a peptide spanning the actin binding domain and first central repeat. In *fliA³* the synthesis of an adult muscle-specific isoform is blocked in flight and leg muscles, while in *fliA⁴* the synthesis of nonmuscle and both muscle-specific isoforms is severely reduced. Affected muscles are weak or paralyzed, and,

in the case of *fliA³*, atrophic. Their myofibrils, while structurally irregular, are remarkably normal considering that they are nearly devoid of a major contractile protein. Also surprising is that no obvious nonmuscle cell abnormalities can be discerned despite the fact that both the *fliA¹*- and *fliA⁴*-associated mutations perturb the nonmuscle isoform. Our observations suggest that α -actinin stabilizes and anchors thin filament arrays, rather than orchestrating their assembly, and further imply that α -actinin function is redundant in both muscle and nonmuscle cells.

THE cellular and developmental roles of the spectrin superfamily of proteins are being clarified by genetics. Hemolytic diseases of humans are known to result from spectrin defects that weaken the erythrocyte plasma membrane, predisposing the cells to collapse in the face of circulatory system shear forces (Knowles et al., 1983; Marchesi et al., 1987). Analyses of humans, dogs, and mice having muscular dystrophies have correlated dystrophin defects with syndromes of muscle wasting (Hoffman et al., 1987; Cooper et al., 1988). These results suggest that mutant dystrophins engender muscle necrosis by failing to localize or anchor sarcolemmal glycoproteins (Ervasti et al., 1990). Genetic investigations of the third spectrin superfamily member and principal actin filament cross-linking protein, α -actinin, have not been as informative. Gene knockout experiments performed in *Dictyostelium* did not confer a detectable phenotype (Wallraff et al., 1986; Noegel and Witke, 1988; Schleicher et al., 1988). This result almost certainly signifies that the role of α -actinin in nonmuscle cells is largely redundant, but does not clarify the function of the protein. α -Actinin mutations in *Drosophila* engender either lethal or flightless phenotypes (Fyrberg et al., 1990). No nonmuscle cell phenotypes have been noted, but within muscles disruptions of Z-discs and attachments of muscle fibers to epithelial tendon cells are readily apparent (Fyrberg et al., 1990).

Our understanding of α -actinin functions in muscle cells remains rather limited. Immunolocalizations of muscle α -actinin have demonstrated conclusively that it is within

Z-discs, where it has been proposed to cross-link ends of both parallel and antiparallel arrays of actin filaments (Suzuki et al., 1976; Endo and Masaki, 1982; Duhaiman and Bamburg, 1984; for review see Blanchard et al., 1989). In vitro studies have shown that at very low (0–4°C) temperatures, antiparallel dimers of α -actinin do indeed cross-link actin filaments (Goll et al., 1972; Bennett et al., 1984), but the length of these cross-links (350 Å) is considerably greater than those of analogues visualized in image reconstructions of bee Z-discs (170–240 Å), suggesting that in vitro and in vivo α -actinin conformations can be rather distinct (Cheng and Deatherage, 1989; Deatherage et al., 1989). One means by which to reconcile these results is to propose that thin filament cross-links are comprised of additional proteins that modify the conformation of α -actinin. In support of this hypothesis, Goll et al. (1990) recently have shown that controlled digestions of Z-discs with the neutral protease calpain release both α -actinin and actin intact. This result may imply that at least one additional protein facilitates binding of actin to α -actinin, and raises questions as to how directly α -actinin participates in cross-link formation.

To evaluate in vivo α -actinin functions and relate them to syndromes of abnormalities we are investigating several *Drosophila fliA* mutants. In this paper, we detail effects of the associated mutations on α -actinin mRNA and protein accumulation, correlating these changes with abnormalities in myofibril morphology. We demonstrate that one exon within the *Drosophila* α -actinin gene is variably spliced to generate one nonmuscle and two muscle isoforms whose primary se-

quence differences are confined to a peptide that spans the actin binding domain and first central repeat. *fliA¹* has a single G>A transition within the variable exon, adjacent to an internal donor site, that prevents splicing of the mRNA specifying the adult muscle-specific isoform. Consequently, the appropriate α -actinin isoform is not expressed within adult fibrillar and tubular muscles, and only a small amount of supercontractile muscle isoform accumulates. Initially the organization of most flight muscle sarcomeres is surprisingly normal, considering that they have severely reduced amounts of a major contractile protein. However, the fibers are paralyzed and myofibrils degenerate as flies age. In *fliA⁴* an A>T transversion within the acceptor site of the last intron severely reduces the accumulation of normal α -actinin mRNA in both muscle and nonmuscle cells. Adult muscles are weak and paralyzed, but there is no discernible associated nonmuscle phenotype. On the basis of comparisons of four *fliA* mutants we conclude that reductions and perturbations of α -actinin have at most only subtle effects on nonmuscle cells and unexpectedly minor effects on myofibril structure. However, these same mutations cause muscle weakness, paralysis, and, in one case, atrophy. Our results are most compatible with the hypothesis that α -actinin reinforces and stabilizes microfilament arrays, rather than orchestrating alterations in their organization during development and morphogenesis.

Materials and Methods

Drosophila Strains

fliA¹, *fliA²*, *fliA³*, and *fliA⁴* strains were obtained from Dr. Ted Homyk (University of Virginia). Stocks were maintained as homozygotes on yeast-cornmeal-agar medium. The Canton-S strain was taken to be wild type in every instance.

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 μ g 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% (wt/vol) SDS. Hybridizing fragments were visualized by exposure to X-ray film.

RNA Preparation, Electrophoresis, and Blotting

RNA was extracted from synchronously developing *Drosophila* cultures by the SDS-phenol technique (Spradling and Mahowald, 1979). Poly(A)⁺-containing RNA was prepared from early (0–4-h), mid (8–12-h), and late (20–24-h) embryo stages; early (145-h), mid (169-h), and late (186-h) pupal stages; Schneider's cells; second instar larvae; and newly eclosed adults. All times are postfertilization at 25°C. 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).

Sequencing of *fliA* Mutations

fliA mutations were sequenced by constructing genomic libraries from DNA of each strain, isolating their α -actinin genes, and then determining sequences of all exons, intron donor sites, and intron acceptor sites. Library construction and screening were done according to standard protocols (Maniatis et al., 1978). DNA sequencing was performed using the dideoxynucleotide method (Sanger et al., 1977), in conjunction with a set of oligonucleotide primer sequences that collectively allowed sequencing of all exons and flanking intronic sequences.

Preparation of Proteins for Gel Electrophoresis

Flight muscles were removed from thoraces and homogenized in buffer O of O'Farrell (1975). To cleanly separate indirect flight muscles (termed "fibrillar muscles") from other thoracic muscles they were shrunken by immersing whole flies in –20°C acetone for 2–3 d before dissection (Mogami et al., 1982). Proteins of other muscle types were obtained by homogenizing heads, legs, or whole thoraces in buffer O. Tissue homogenates were boiled for 2 min and then centrifuged at 12,000 g for 30 s. Bromphenol blue was added to samples, and proteins were electrophoresed on 10% SDS-polyacrylamide gels according to Laemmli (1970).

Immunoblotting

Gels were rinsed for 15 min in transfer buffer (0.0125 M Tris, pH 10.5, 0.096 M glycine) containing 0.1% SDS, and then electroblotted 24 h at 150 V according to Towbin et al. (1979). After transfer, filters were rinsed in deionized water and stored for at least 1 h in blocking solution (50 mM Tris, pH 7.4, 3% wt/vol BSA, 0.9% wt/vol NaCl, 0.04% wt/vol sodium azide) at room temperature or 4°C. Blots were incubated for 2 h at room temperature with either a monoclonal antibody raised against waterbug α -actinin (provided by Dr. B. Bullard) or a polyclonal antibody recognizing chicken α -actinin (provided by Dr. K. Burrige), and then for 1 h with a 1:200 dilution of peroxidase-conjugated rabbit anti-rat or goat anti-rabbit secondary antibody. Secondary antibody binding was visualized by developing blots in a solution containing 1 part 3 mg/ml chloronaphthol in methanol and 5 parts TBS (50 mM Tris, pH 7.5, 150 mM NaCl) plus 0.5% vol/vol hydrogen peroxide, or using the enhanced chemiluminescence system marketed by Amersham Corp. (Arlington Heights, IL).

Isolation of α -Actinin cDNAs

Early embryo and larval cDNA libraries (kindly provided by T. Kornberg; refer to Poole et al., 1985) were screened with a *Drosophila* α -actinin mid-pupal stage cDNA. Isolated cDNAs were subcloned in the pUC19 vector and sequenced using the method of Sanger et al. (1977).

Polymerase Chain Reaction Amplification of α -Actinin cDNAs

α -Actinin cDNAs were synthesized from staged poly(A)⁺ RNA preparations of wild-type and *fliA³* flies using the polymerase chain reaction (PCR)¹ method. 2 μ g of poly(A)⁺ RNA was denatured by heating to 70°C, annealed to 10 μ g/ml of an appropriate antisense oligonucleotide primer, and transcribed using highly purified reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 90 min at 37°C. The cDNA product was heated to 95°C for 5 min, and 25% was amplified as described below. 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 μ g each in a final volume of 100 μ l), and the mixture was incubated for 30 cycles of denaturation, renaturation, and extension (94°/1 min, 55°/1 min, and 72°/3 min, respectively) in the presence of highly purified Taq polymerase. The following primers were used: codons 228–235 (sense orientation) of the nonmuscle alternative exon – 5' ATCTC-CAGAATACAGCTTTGC 3'; codons 228–234 (sense orientation) of the muscle alternative exon – 5' ATTTGATCAACACTCCGAAACC 3'; codons 307–314 (antisense orientation, referred to herein as the codon 300 primer) – 5' CTCTGACACCTGCCAGCGAGTT 3'; codons 819–825 (sense orientation) – 5' ATGACCCGCGAGACACCGAT 3'; and 3' untranslated region (antisense orientation and incorporating a Sall restriction site) – 5' GGTCGACCTTCGGATCGGGTGGTGCC 3'.

Recovered cDNA products were analyzed by electrophoresis in 1.5% agarose. To determine the nucleotide sequences of amplified products one strand was selectively replicated using asymmetric PCR, and then sequenced using the method of Sanger et al. (1977). Asymmetric PCR was performed like the standard PCR protocol, except that the limiting primer was present in only 1/50th the concentration of the nonlimiting primer, and twice as much Taq polymerase was used. The selectively amplified strand was annealed to the limiting primer and sequenced using the method of Sanger et al. (1977).

1. Abbreviation used in this paper: PCR, polymerase chain reaction.

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 KMOPS (pH 6.8) at 4°C overnight. Hemithoraces were rinsed once with buffered Ringer's, and then twice with 0.1 M NaPO₄, 10 mM MgCl₂ (pH 6.0). Hemithoraces 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 a 50, 70, 95, and 100% ethanol series. After infiltration with Epon 812/dodecylsuccinic anhydride/araldite (1:7:2 by vol), fibers were removed by dissection, placed in a drop of resin containing catalyst, and the resin was polymerized by incubation at 45°C for 12 h and 70°C for 12 h. Silver-gold sections were cut, stained with uranyl acetate and lead citrate, and observed in the electron microscope.

Results

A Single *Drosophila* α -Actinin Gene Specifies Nonmuscle, Larval Muscle, and Adult Muscle Isoforms

We have previously reported the isolation of a *Drosophila* α -actinin gene, located within the 2C subdivision of the X chromosome, and correlated it with both *lethal(1)2Cb* and *fliA* mutations (Fyrberg et al., 1990). To elaborate how these mutant alleles cause phenotypic abnormalities it was essential to establish whether isoforms of *Drosophila* α -actinin existed, and to document how they were specified. We obtained evidence that the 2C subdivision gene encoded both nonmuscle and muscle-specific α -actinin mRNAs by hybridizing a cDNA probe to a developmentally staged series of *Drosophila* RNAs (Fig. 1; see Materials and Methods for staging of embryos and pupae). We observed strong hybridization both to stages having (mid-to-late embryos, larvae, and mid-to-late pupae) and lacking (early embryos and early pupae) developing muscle fibers. We emphasize that the embryonic muscle pattern is not prefigured until 5.5 h after fertilization, hence 0–4-h embryos have no differentiating myoblasts (Bate, 1990). Likewise, while no stage of pupation lacks myoblasts completely, 145-h postfertilization (25-h postpupariation) pupae have little muscle tissue because larval body wall fibers have been histolyzed and adult muscle fibers have not yet appeared (Fernandes et al., 1991). To ensure that the staged animals lacking muscles were not contaminated with a small percentage of older animals having developing muscles, the α -actinin probe was removed by boiling, and the blot rehybridized with a muscle-specific (troponin T) probe. No trace of hybridization could be detected in either early embryos or early pupae, demonstrating that animals in these preparations were staged properly and were indeed devoid, or nearly devoid, of muscles. Our finding that the same α -actinin gene encodes both nonmuscle and muscle-specific mRNAs, taken together with our failure to isolate related *Drosophila* genes or cDNAs during an extensive series of low-stringency hybridization screens of libraries with the most highly conserved portion of the original cDNA, suggested that a single *Drosophila* α -actinin gene encoded both nonmuscle and muscle-specific isoforms.

To verify that distinct nonmuscle and muscle α -actinin isoforms were encoded by a single gene, we compared the sequence of a cDNA isolated from pupae having developing adult muscles to one isolated from early embryos collected before the initiation of muscle formation. Sequence differences were confined to codons 229–257, which specify a

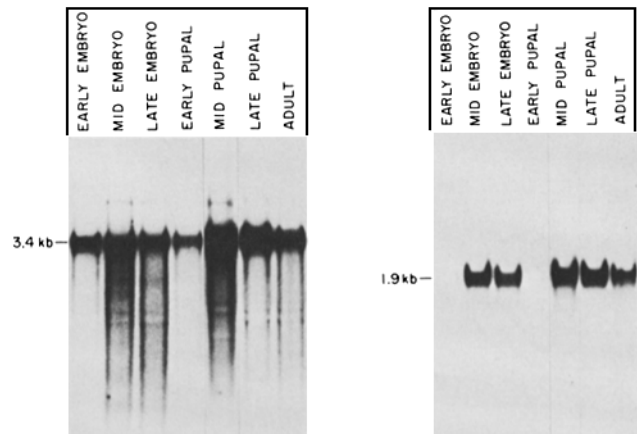


Figure 1. Developmental profile of *Drosophila* α -actinin mRNA accumulation. Poly(A)⁺ mRNA extracted from the illustrated stages (refer to Materials and Methods for the precise staging) was reacted with a ³²P-labeled α -actinin cDNA (left). mRNA having the expected size was seen in all stages, including early embryos and early pupae, which lack myoblasts and developing muscle fibers, respectively. To be certain that hybridization during these stages was not due to contamination of these preparations with animals having developing muscles, the blot was stripped of the original probe and rehybridized to a muscle-specific gene (troponin T). No hybridization to the 1.9-kb mRNA was seen in early embryo and early pupal preparations, confirming that they do not contain mRNA derived from developing muscles.

peptide located at the junction of the actin binding domain and the first central repeat (see Blanchard et al., 1989; Fyrberg et al., 1990). Localization of the corresponding genomic DNA revealed that the distinct sequences were specified by two alternatively spliced exons, as depicted in Fig. 2, the muscle-specific exon being located 5' to that specifying the nonmuscle sequence.

We found additional heterogeneity within the muscle-specific exon when we investigated the sequence of larval α -actinin cDNA. Larval cDNA fragments that included the muscle-specific exon were noted to be larger than their nonmuscle and pupal muscle counterparts. We discovered that both larval and some adult muscle α -actinin mRNAs specify the insertion of 22 residues after Gln257. The larger mRNA is generated by recognition of a splice donor site located 66 nucleotides downstream from that utilized within adult tubular and fibrillar muscles (see the hatched region in Figs. 2 and 6). The sequences of the junction peptides encoded by nonmuscle, larval muscle, and adult muscle cells are shown in Fig. 3. The nonmuscle and adult muscle peptides are 72% identical. The *Drosophila* nonmuscle isoform sequence is more similar to chicken fibroblast/smooth muscle α -actinin (21 of 29 residues identical; see Arimura et al. [1988] for the chicken α -actinin sequences) than to the chicken skeletal muscle isoform (18 of 29 residues identical). Likewise, the *Drosophila* adult muscle isoform peptide is more similar to the chicken skeletal muscle isoform (24 of 29 residues identical) than to the fibroblast/smooth muscle isoform (19 of 29 residues identical). These comparisons suggest that functional differences between muscle and nonmuscle α -actinin isoforms have structural correlates within the variably spliced peptide that extend over the evolutionary distance separating invertebrates and vertebrates. The 22 residues in-

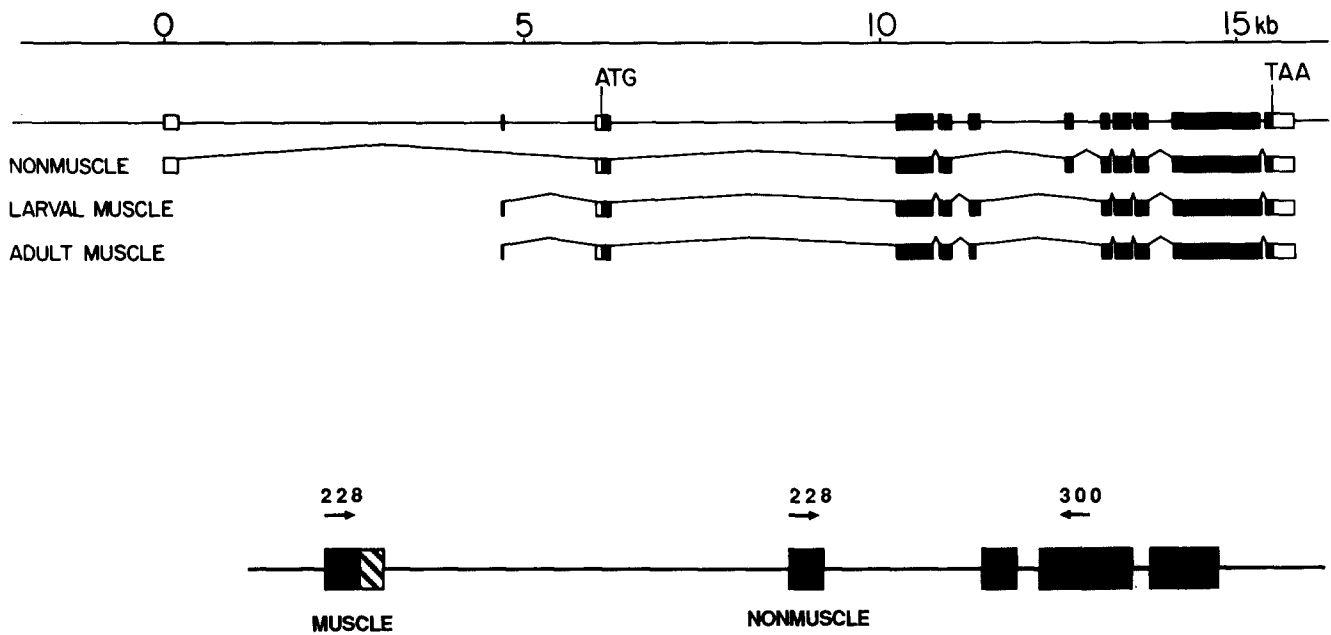


Figure 2. Schematic representation of alternatively spliced *Drosophila* α -actinin mRNAs. The upper line illustrates the chromosomal arrangement of exons in the *Drosophila* α -actinin gene. Solid and hatched blocks represent protein coding regions, while open blocks denote untranslated regions. Directly below we show the structures of nonmuscle, larval (supercontractile) muscle, and adult muscle mRNAs, as deduced from cDNA sequences. mRNA recovered from nonmuscle sources (Schneider's cells, early embryos, early pupae) includes the distal 5' untranslated region exon and a protein-encoding exon (including codons 229–257) that is not utilized in muscle cells. Larval and adult supercontractile muscles transcribe the more proximal 5' untranslated exon and a distinct coding region exon that is 22 codons longer than, but otherwise identical to, that utilized in adult tubular and fibrillar muscles. In the lower portion of the diagram we show an expanded diagram of the alternately spliced muscle and nonmuscle exons. Directly above the exons we show the locations of oligonucleotide primers used to amplify portions of cDNAs.

serted within the larval isoform are not highly similar to any α -actinin sequence, or any peptide sequence present in sequence databases. We note, however, that the larval peptide contains three proline residues, and therefore must adopt an open, or nonhelical, conformation.

The observed heterogeneity in the larval and adult α -actinin mRNA and protein sizes corroborates immunoblotting analyses of Z-disc protein distribution in various *Drosophila* muscles reported by Vigoreaux et al. (1991). They found two distinct sizes of α -actinin in *Drosophila* muscles. The larger of the pair predominates in larval body wall and adult muscles of heads and abdomens, but is absent from adult indirect flight muscles and tubular jump and leg muscles. Hence, their evidence strongly suggests that the more slowly migrating α -actinin isoform is found within supercontractile muscles of the larva and adult, fibers having perforated Z-bands through which thick filaments protrude during extreme shortening (Crossley, 1978). The more rapidly migrating α -actinin isoform specifically accumulates within adult fibrillar and tubular muscles.

	230	240	250	260	270
NONMUSCLE:	DLQNTALPDERAVMTYVSSYYHCFSGAQK				
LARVAL MUSCLE:	DLINTPKPDERAIMTYVSCYYHAFQGAQQVGNVTHVPEPTRQYTYVPNNYN				
ADULT MUSCLE:	DLINTPKPDERAIMTYVSCYYHAFQGAQQ				

mRNAs are 72% identical. The extra 22 residues specified by the supercontractile muscle mRNA are not highly similar to any sequence currently entered in DNA databases, including primary sequences for several α -actinin isoforms of various organisms. Note, however, that 3 of 22 residues are proline, and consequently that the peptide is unlikely to adopt a helical conformation. These sequence data are available from EMBL GenBank/DDJB under accession number X51753.

*fliA*³ and *fliA*⁴ Mutants Fail to Accumulate Normal Levels of α -Actinin

To correlate phenotypic abnormalities with α -actinin defects we have analyzed four *fliA* mutant strains. *fliA* mutants are flightless, due to abnormalities within their indirect flight muscles. They fail to complement *lethal(1)2Cb* mutants having chromosome rearrangements within their α -actinin genes, suggesting that both the lethal and flightless syndromes are due to mutations of the same α -actinin gene (Homyk and Emerson, 1988; Fyrberg et al., 1990; our unpublished results). Southern blot analyses of *fliA* mutants did not reveal any DNA rearrangements within the α -actinin gene (data not shown), however, and all were presumed to have point mutations.

We began our investigation of *fliA* mutants by examining the accumulation of α -actinin using Western blots. We dissected indirect flight muscles from newly eclosed adults, and separated component proteins using SDS-PAGE. Proteins were transferred to nitrocellulose and reacted with either

Figure 3. Comparisons of primary sequences encoded by nonmuscle, larval muscle, and adult muscle α -actinin mRNAs. Primary sequences specified by codons 229–257 of nonmuscle and muscle

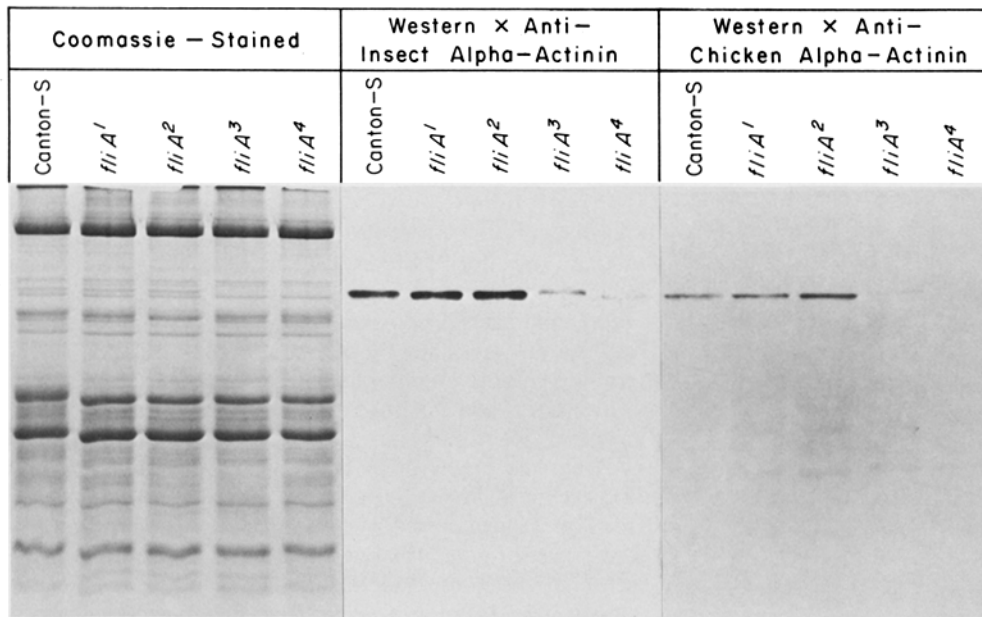


Figure 4. Western blot analysis of α -actinin accumulation in *fliA* flight muscles. The left-hand panel shows Coomassie blue-stained proteins from isolated flight muscles of wild-type (Canton-S) and *fliA* mutants. The middle panel is a Western blot of the same preparation reacted with a monoclonal antibody raised against waterbug α -actinin. Note the slightly elevated level of α -actinin in *fliA*², and the virtual absence of reactive protein in *fliA*³ and *fliA*⁴ mutants. The right panel is a duplicate Western blot reacted with a polyclonal antibody raised against chicken α -actinin. Again, very little α -actinin can be detected in *fliA*³ and *fliA*⁴ flight muscles.

a monoclonal antibody recognizing waterbug α -actinin (provided by Dr. Belinda Bullard), or a polyclonal antibody raised against chicken α -actinin (provided by Dr. Keith Burridge). As can be seen in Fig. 4, the reactions of both antibodies were identical. We found normal levels of α -actinin in *fliA*¹ flight muscles, slightly increased levels of α -actinin in *fliA*², and only barely detectable amounts in *fliA*³ and *fliA*⁴ flight muscles. We also noted that the small amount of *fliA*³ product has a slightly greater apparent molecular weight than that of Canton-S or the other *fliA* mutants, suggesting that *fliA*³ mutants synthesize the supercontractile muscle isoform in flight muscles.

Western blots of proteins isolated from other types of *Drosophila* muscles demonstrated that *fliA*³ abnormalities are limited to fibrillar and tubular flight and leg muscles, while *fliA*⁴ apparently has reduced α -actinin accumulation in all adult muscles (refer to Fig. 5). Neither α -actinin accumula-

tion nor its apparent molecular size was affected in head muscles of *fliA*³ mutants, while the *fliA*⁴ protein was sharply reduced. In legs, which contain only tubular muscles, results were essentially identical to those seen in flight muscles, except that a doublet of α -actinin is clearly visible in *fliA*³. In whole thoraces, which contain fibrillar and tubular muscles, both *fliA*³ and *fliA*⁴ have α -actinin reductions, and a doublet of *fliA*³ product is again apparent. In neither whole thoraces nor legs does the loss of α -actinin in *fliA*³ mutants appear to be as severe as in fibrillar indirect flight muscles, indicating that the mutant α -actinin is more efficiently synthesized or inherently more stable in tubular muscles than within fibrillar flight muscles. In sum, the series of Western blots revealed that *fliA*³ mutants have reduced levels of more slowly migrating α -actinin within adult fibrillar muscles, and reduced levels of a more slowly migrating α -actinin doublet in tubular muscles; other tissues appear normal. In contrast,

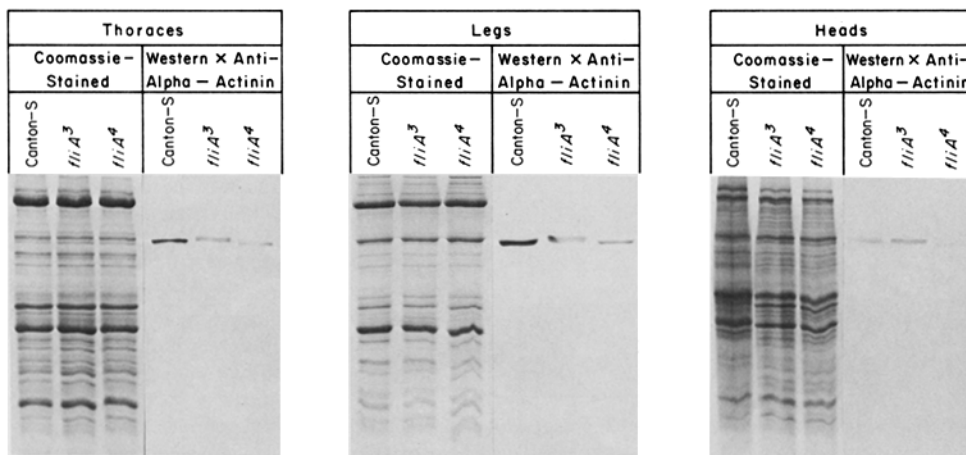


Figure 5. Western blot analysis of α -actinin accumulation in various adult muscles. Thoraces, legs, and heads were homogenized, and component proteins separated by SDS-PAGE. Each of these preparations contains both muscle and nonmuscle tissues, but α -actinin is considerably more abundant in muscle cells than in nonmuscle, and in our experiments peroxidase-conjugated secondary antibodies typically reveal only muscle protein unless more sensitive methods are used. Proteins were transferred to nitrocellulose and

reacted with the anti-waterbug α -actinin. In thoraces, reduced levels of an apparent doublet of more slowly migrating protein accumulate in *fliA*³, and severely reduced levels of protein accumulate in *fliA*⁴. More or less the same result is seen in legs. Head muscles appear unaffected in *fliA*³, while α -actinin is severely reduced in *fliA*⁴.

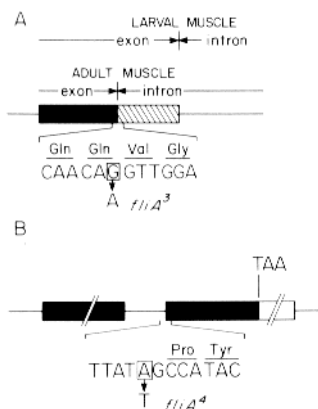


Figure 6. Sequence alterations in *fliA³* and *fliA⁴* α -actinin genes. (A) The variably spliced muscle-specific exon, with the exon-intron borders for the larval muscle and adult muscle mRNAs indicated. In *fliA³* the G of the glutamine codon is converted to A. This alteration changes the context of the adjacent intron donor sequence. (B) In *fliA⁴* the A of the acceptor site of the 3' intron is changed to T.

fliA⁴ mutants have sharply reduced levels of α -actinin in all muscles examined, and, as we show below, in nonmuscle tissues as well (see Figs. 8 and 9).

fliA³ and *fliA⁴* Synthetic Failures Are Due to Splicing Defects

To precisely define the α -actinin gene defects in each *fliA* mutant we sequenced all exons, as well as intron donor and acceptor sites, of each allele. In *fliA¹* and *fliA²* the identities of single codons were altered. In *fliA¹* glycine86 is converted to serine, while in *fliA²* serine 245 is converted to phenylalanine (data not shown). The effects of these mutations on α -actinin function are not yet known. The *fliA¹* mutation is within a constitutively utilized exon that encodes a portion of the actin binding domain, and must hence affect α -actinin function, most likely actin binding, in both nonmuscle and muscle cells. The *fliA²* mutation is within the muscle-specific alternative exon, and accordingly should disrupt functioning of this peptide in muscle, but not affect nonmuscle tissues. From Western blots (see Fig. 4), it is clear that nei-

ther mutation prevents α -actinin accumulation during myogenesis.

In both *fliA³* and *fliA⁴* we found mutations that could potentially affect splicing. In *fliA³* the third base of the glutamine codon that immediately precedes the alternatively utilized internal intron donor site of adult muscle (Fig. 6 A) is changed from G to A. As we show below, this mutation abolishes production of the isoform characteristic of adult tubular and fibrillar muscles. In *fliA⁴* the penultimate nucleotide of the last intron is changed from A to T. This mutation eliminates 3' portions of all *Drosophila* α -actinin mRNAs, and must alter the sequence of the α -actinin COOH terminus in both nonmuscle and muscle cells (see Fig. 8).

To verify that the *fliA³* and *fliA⁴* mutations affected α -actinin RNA splicing we PCR-amplified portions of cDNAs representing mRNAs of various *Drosophila* stages. In the case of *fliA³* we used three oligonucleotide primers (see Fig. 2), one hybridizing to the 5' end of the nonmuscle exon (sense orientation), one to the 5' end of the muscle exon (sense orientation), and one to codon 300 (specifically, codons 307-314) of a constitutively utilized exon located 3' to the alternate exon (antisense orientation). The results of experiments performed using these primers are summarized in Fig. 7. The top portion of the figure illustrates amplifications of wild-type (Canton-S) RNA. When we amplify cDNA using a sense primer that includes codon 228 at the start of the nonmuscle variable exon, and an antisense primer including codons 307-314 (codon 300 primer), we find the expected 234-nucleotide pair band in all stages as well as in Schneider's cells, consistent with cytoskeletal, rather than muscle-specific, function. In late embryo and larval preparations we occasionally detect a second band of 300 nucleotides. This observation may indicate that an as-yet-undocumented exon is utilized in some cytoskeletal transcripts. However, several attempts to sequence asymmetrically amplified transcripts of this band in our laboratory have failed,

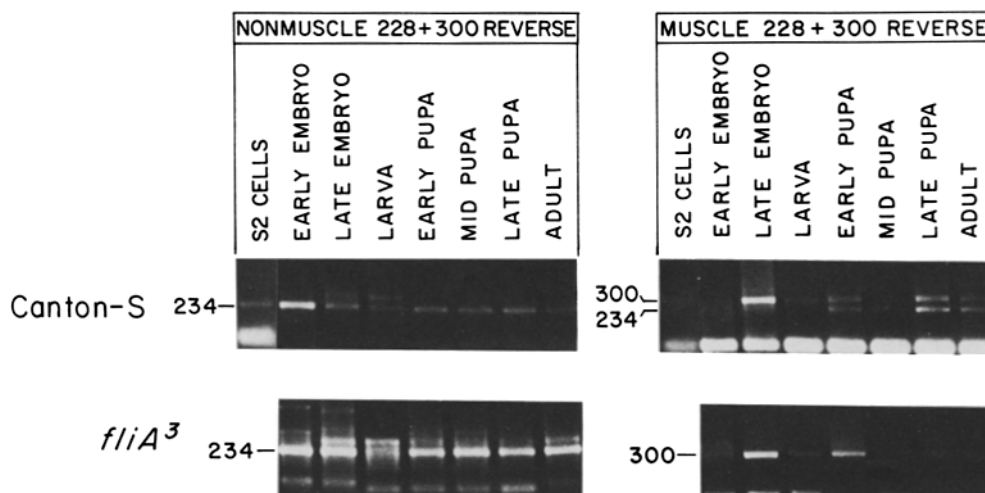


Figure 7. PCR analyses of alternative *Drosophila* α -actinin mRNA splicing in wild-type and mutant strains. To examine the utilization of alternative protein coding exons, an oligonucleotide primer recognizing codons 228-235 within the 5' region of the putative nonmuscle coding region alternative exon was used to amplify cDNA in conjunction with an antisense primer bound to codons 307-314. A band of the expected size is recovered from all developmental stages, as well as Schneider's cells, consistent with a nonmuscle role. In the converse experiment the

5' primer annealed to codons 228-235 of the muscle exon. Bands of 300 and 234 nucleotides were recovered only in stages having developing muscles. Based upon our immunoblotting analyses and those of Vigoreaux et al. (1991), we believe that the larger mRNA sequence typically accumulates only in larval and adult supercontractile muscles, while the lower band accumulates within adult tubular and fibrillar muscles. The lower portion of the figure illustrates an identical experiment performed using temporally staged *fliA³* mRNAs. The accumulation of the nonmuscle and larval muscle mRNAs are unaffected, but no adult tubular/fibrillar mRNA can be detected. Bands at the bottom of each lane are unhybridized primers.

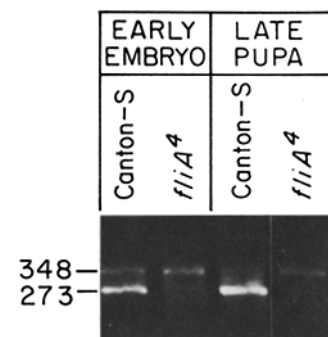


Figure 8. PCR analyses of 3' α -actinin mRNA sequences in wild-type and *fliA⁴* early embryos and late pupae. The figure illustrates results of PCR amplifications of early embryo and late pupal RNA of wild type and *fliA⁴* using a pair of primers that span the last intron. In both stages the expected 273-nucleotide band is present in wild type and absent from *fliA⁴*, demonstrat-

ing that one or both regions to which these primers anneal is absent from *fliA⁴* mRNA. The 348-nucleotide band present in *fliA⁴* and some Canton-S preparations is apparently amplified because of genomic DNA contaminating the mRNA preparations, since pretreatment of mRNA with DNase eliminates it, and also because it can be amplified without prior reverse transcription of mRNA.

suggesting that it is not derived from *Drosophila* α -actinin mRNA.

When we used the same 3' primer just described in conjunction with one annealing to codon 228 of the muscle-specific variable exon, we found that two products accumulated during stages when *Drosophila* muscles develop. The more slowly migrating 300-nucleotide band was found to include the 22 codons present in larval mRNA. That this same band can be amplified from pupal stages demonstrates that these codons are included in some adult muscle mRNAs, almost certainly those found within supercontractile muscles of the head and abdomen (see Fig. 5 of this manuscript; Vigoreaux et al., 1991). DNA sequencing of the more quickly migrating band has demonstrated that it contains the muscle-specific sequence without the 22 codons. These observations validate the alternative splicing pattern illustrated in Figs. 2 and 6, and serve as a reference to which to compare mutants.

We next examined the pattern of amplified fragments seen in *fliA³* mutants. An identical series of staged mRNAs was prepared and annealed with the same three primers. Results are illustrated in the lower portion of Fig. 7. Accumulation of the nonmuscle sequence is unaffected, as we expected from the fact that the mutation is within the muscle-specific exon. When amplifications were performed using the muscle exon sequence we found that only the more slowly migrating supercontractile muscle sequence is present; none of the adult fibrillar/tubular muscle cDNA can be discerned. To confirm the identity of the 300-nucleotide pair band, we determined its sequence and found that it indeed included the extra 22 codons of supercontractile muscle mRNA. Thus, muscle-specific splicing of *fliA³* α -actinin mRNA is limited to the supercontractile muscle mode, and as a consequence adult tubular and fibrillar muscles do not synthesize the correct isoform. Western blots illustrated in Figs. 4 and 5 suggest that a more slowly migrating protein, which we believe is identical to the larval isoform, accumulates to low levels in flight and leg muscles; whether the second reactive protein seen in tubular muscles of the thorax and leg is a proteolytic product or aberrant protein is not yet known.

To evaluate the effects of the *fliA⁴* mutation we prepared a set of oligonucleotide primers that spanned the most 3' intron, and amplified the intervening mRNA sequence. Before undertaking PCR analyses, we used Northern blots of *fliA⁴*

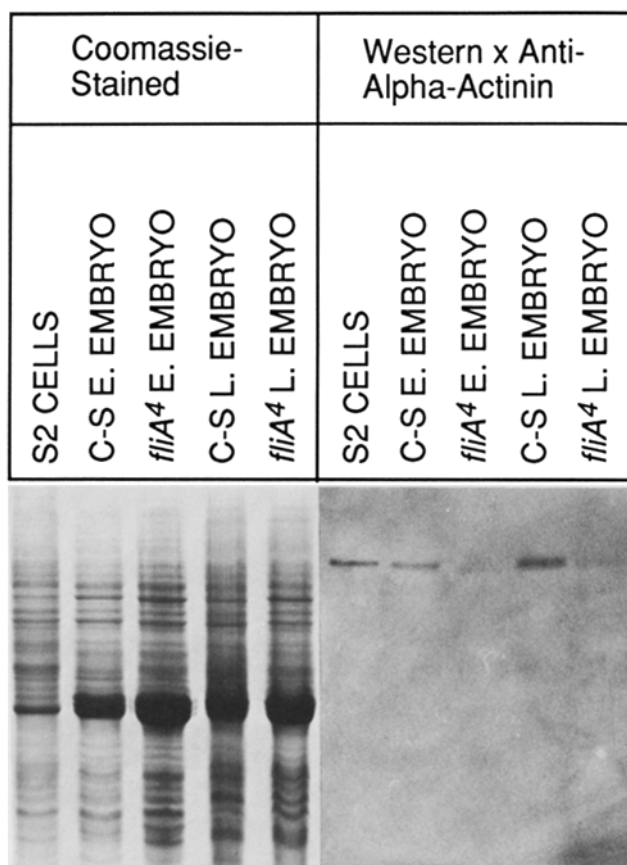
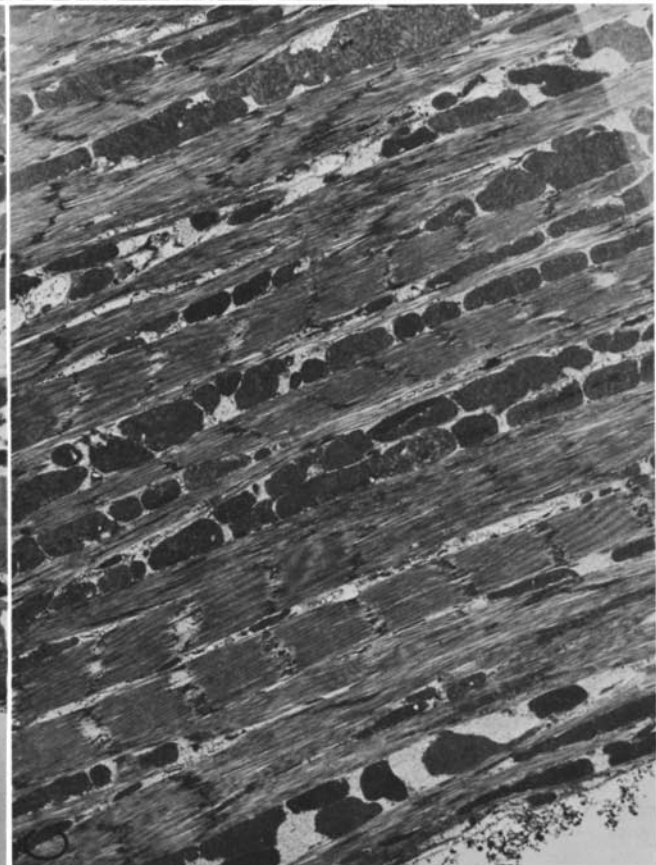
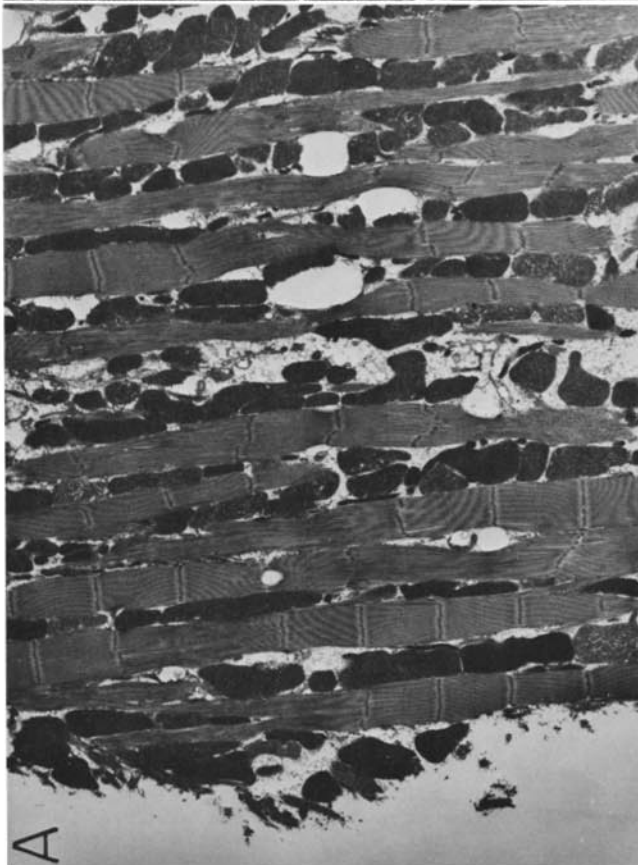
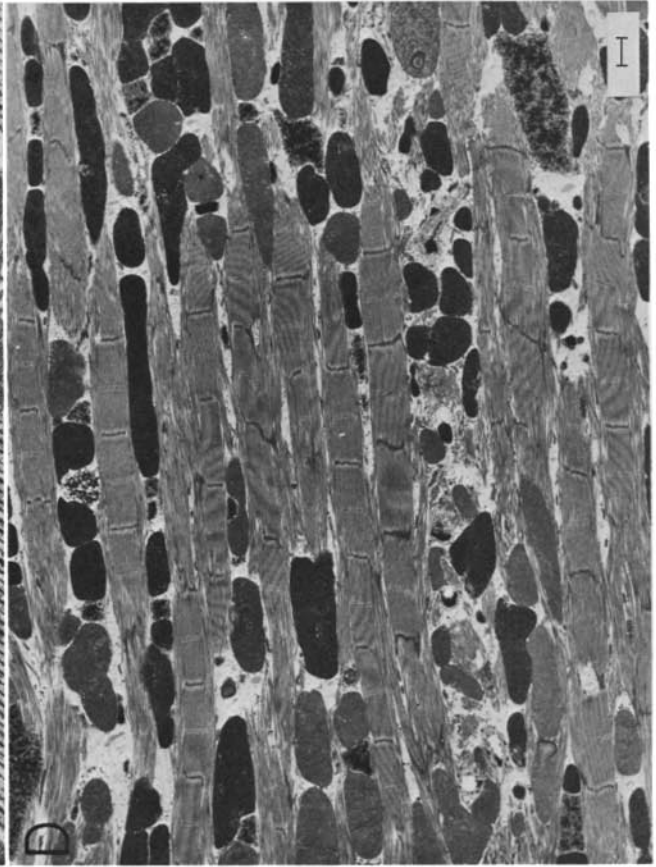
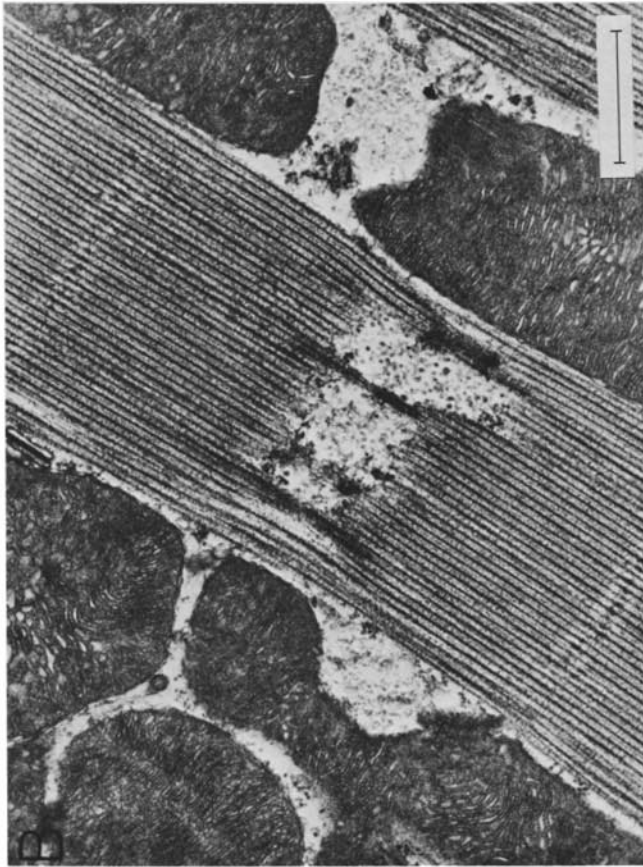


Figure 9. Western blots of α -actinin from wild-type and *fliA⁴* embryos. The left half of the figure illustrates Coomassie-stained proteins of Schneider cells, Canton-S early embryos (aged 2 h), *fliA⁴* early embryos, Canton-S late embryos (aged 18 h), and *fliA⁴* late embryos. In the right panel, proteins were reacted with either of the anti- α -actinin antibodies described in the text. Marked reductions of α -actinin in *fliA⁴* preparations were seen using either antibody, demonstrating that the mutation lowers accumulation of non-muscle and larval muscle isoforms.

pupal mRNA to demonstrate that normal levels of α -actinin message are present, but the mRNA is substantially larger than that seen in wild-type flies (data not shown). Comparison of wild-type and *fliA⁴* PCR products (Fig. 8) revealed that in both early embryos and pupae the expected 273-nucleotide band is sharply reduced or absent. This result demonstrates that *fliA⁴* mRNA lacks all or part of the intervening DNA, which includes codons 819–897. We find a band of 348 nucleotides in some *fliA⁴* and Canton-S preparations. We have sequenced this band and shown that it includes codons 819–897 as well as the 75-nucleotide intron. Most or all of this product appears to be copied from small amounts of genomic DNA that copurify with mRNA, because it can be amplified without prior reverse transcription of RNA or eliminated by pretreatment of RNA with DNase (data not shown). We emphasize, however, that the recovery of this band demonstrates that primers used in this experiment anneal to *fliA⁴* DNA and are therefore competent to amplify corresponding mRNA sequences. In experiments that have used three additional sense primers that anneal 5' to the intron, we have conclusively demonstrated that portions of *fliA⁴* α -actinin mRNAs upstream of this intron are



missing, and consequently that the mRNA cannot specify synthesis of normal α -actinin isoforms (data not shown). However, it is possible that the presumed splicing defect is suppressed at least occasionally, and that a small amount of normal protein is produced, as the Western blots shown in Figs. 4 and 5 suggest.

To more rigorously establish that *fliA*⁴ nonmuscle cells have severely reduced levels of α -actinin, we reacted early embryo and late embryo proteins with both antibodies described in the legend to Fig. 4. Antigen could be readily detected in Schneider-2 cells, and in Canton-S early and late embryos. However, only small amounts could be detected in early or late *fliA*⁴ embryos using either antibody (Fig. 9). We note that one of these antibodies is a polyclonal raised against chicken α -actinin and, therefore, might be expected to be broadly cross-reactive. The failure of this antibody to detect normal levels of α -actinin in *fliA*⁴ early embryos strongly suggests that the α -actinin gene described here is the only *Drosophila* gene that specifies this protein in nonmuscle cells.

Assembly and Maintenance of *fliA*³ Myofibrils

To extend our initial analyses of *fliA* muscle defects (see Fryberg et al., 1990), we have used transmission electron microscopy to evaluate sarcomeric organization in both young (2-d posteclosion) and old (17-d posteclosion) *fliA* mutants. Although abnormalities are readily apparent, myofibrillar lattices of recently eclosed *fliA*³ flight muscles are remarkably normal (Fig. 10 A), considering that they contain so little α -actinin. Most sarcomeres consist of well-arranged thick and thin filaments, as evidenced by the regular occurrence of M-lines and Z-discs. In transverse sections of myofibrils most thick and thin filaments are precisely packed in double hexagonal arrangement (data not shown). Z-discs are occasionally discontinuous, or punctate, consistent with the fact that α -actinin is an integral component, and M-lines are not straight, suggesting that the lateral registration of thick and thin filaments is imperfect. These abnormalities evidently suffice to preclude contraction, as *fliA*³ flight muscles are paralyzed and mutants are flightless. Mutant homozygotes also jump poorly, and have difficulty walking vertically, demonstrating that functioning of tubular leg muscles is impaired, consistent with reductions of α -actinin seen in these fibers (see Fig. 5). These observations almost certainly signify that perturbations of myofibrillar structure are sufficient to paralyze flight muscles, but only impair the function of jump and leg muscles. The ability of mutant jump and leg muscles to contract may be attributable to the reduced amounts of α -actinin within them, or to other proteins that serve an analogous thin filament-linking function.

When older *fliA*³ flies are examined, it is clear that severe

degeneration of myofibrils, and in particular of Z-discs, has occurred. Fig. 10, B and C, illustrates longitudinal sections of flight muscles from mutant adults aged 17 d. In dorso-lateral fibers the most prominent difference, in comparison to muscles of younger *fliA*³ mutants, is the dissolution of Z-discs (Fig. 10 B). M-lines have also disappeared, demonstrating that thick and thin filaments have become misregistered, possibly by sliding uncontrollably with respect to one another. We have noted these same defects in tubular jump and leg muscles (data not shown). In dorsoventral flight muscles we frequently find sarcomeres wherein Z-discs are essentially absent, yet thick and thin filaments remain well registered (Fig. 10 C). When whole dorso-lateral flight muscle fibers are viewed in the dissecting microscope, their normal smooth cylindrical shape is interrupted by constrictions indicative of atrophy (data not shown). Despite viewing a large number of sections cut from young *fliA*³ flies, we have never seen such defects in any of their flight muscles. It is clear that *fliA*³ flight muscles profoundly degenerate during the first 2 wk after eclosion, either because myofibrils cannot bear the requisite mechanical loads, or because the mutant myofibrils are subject to necrosis. The breakdown probably is not due merely to reductions of α -actinin, because myofibrils of 17-d-old *fliA*⁴ mutants are stable despite the severity of both nonmuscle and muscle α -actinin deficits (Fig. 10 D). This observation probably signifies that it is a qualitative property of *fliA*³ α -actinin, rather than the reduction of α -actinin, that causes myofibrillar breakdown and fiber atrophy.

Discussion

α -Actinin is the principal and most studied actin filament cross-linking protein (for review see Blanchard et al., 1989), but its functions in muscle and nonmuscle cells remain largely conjectural. To further elaborate its *in vivo* roles we are correlating particular α -actinin defects with syndromes of abnormalities that they engender. Here we report the characterization of four *Drosophila* α -actinin mutations. Two of the four, those associated with *fliA*³ and *fliA*⁴, are especially informative because they cause splicing defects that eliminate particular α -actinin isoforms. In *fliA*³ a single nucleotide alteration abolishes expression of the appropriate α -actinin isoform within adult flight and leg muscles. In flight muscles, where low levels of presumed supercontractile muscle isoform accumulate, paralysis and atrophy ensue. In tubular muscles of the leg the effects on contraction are not as severe, and the main consequence of the mutation is muscle weakness, as evidenced by the inability of flies to jump (see Homyk and Emerson, 1988). The *fliA*⁴ splicing defect markedly reduces the accumulation of both muscle and non-

Figure 10. Electron micrographs of *fliA*³ and *fliA*⁴ flight muscles. A illustrates flight muscle myofibrils of recently eclosed *fliA*³ homozygotes. Z-discs are somewhat thin and punctate, and M-lines are occasionally discontinuous, but overall sarcomere morphology is surprisingly normal. Note in particular that thick and thin filaments are in transverse register, as evidenced by the regular spacing of Z-discs and M-lines. B and C show comparable sections of 17-d-old flight muscles. Skewing and dissolution of the Z-discs are readily apparent (B and C). At low magnification (B), it can be seen that nearly all Z-discs are poorly organized, and thick and thin filaments frequently are not in register. Occasionally we find sarcomeres wherein the Z-disc has degenerated, but thick and thin filaments are intact (C). D illustrates 17-d-old *fliA*⁴ sarcomeres. No myofibrillar degeneration can be seen, demonstrating that *fliA*³ atrophy is not due to the mere reduction of α -actinin. Bars: (A, B, and D) 1 μ m; (C) 0.5 μ m.

muscle α -actinin mRNAs. We can discern no obvious effect of this mutation in nonmuscle tissues; embryogenesis appears normal, as does adult morphology. However, adult muscles are weakened and paralyzed. Our observations support the hypothesis that α -actinin cross-links play an essential role in anchoring and stabilizing thin filaments in muscles, but do not illuminate the functions of nonmuscle α -actinin isoforms. To document whether there are indeed subtle nonmuscle phenotypes in *fliA*⁺ mutants, we have begun to analyze in detail the structure and biology of their nonmuscle cells.

The α -actinin gene described here almost certainly encodes all isoforms of *Drosophila*. We have conducted an extensive search for related genes by hybridizing, at low stringency, the most highly conserved portion of the α -actinin gene described here to *Drosophila* genomic and cDNA libraries and have not found closely related sequences. Were there other *Drosophila* genes encoding α -actinin they would be expected to crosshybridize with this probe, based upon results of Schulze et al. (1989). These authors have shown that a comparable fragment of the *Dictyostelium* α -actinin gene hybridizes efficiently even to mRNA encoding distantly related rat α -actinin. On the basis of our library screens, and taking into consideration the observation that very little α -actinin can be detected in *fliA*⁺ embryos using a polyclonal anti-chicken α -actinin antibody, we are reasonably certain that the α -actinin gene described here is unique.

Remarkably, the only primary sequence differences specified by the three distinct cDNAs described here occur within a short peptide that spans the actin binding domain and first central repeat. Other portions of the primary sequence, including the bulk of the actin binding domain, central repeats 2, 3, and 4, and the EF hand-like sequences, are invariant. Hence, the three encoded α -actinin isoforms must be adapted for their tissue-specific roles by the sequences of the junction peptide, and in the most extreme instance by the eight residues that differ between the nonmuscle and adult tubular/fibrillar muscle isoforms. This peptide may be well placed to modulate molecular functions. It may serve as a hinge between the actin binding head domain and the "rod" portion of the molecule formed by the internal repeat segments (see Blanchard et al., 1989; Koenig and Kunkel, 1990). Alternatively, the conformation of the head may be strongly determined by the character of the junction peptide. It should be noted also that the peptide could well moderate the influences of calcium binding to EF hand-like sequences located within the COOH terminus (residues 752–821), since they may be closely apposed to it in the native antiparallel dimers (see Fig. 1 in Blanchard et al., 1989). Further speculation must await more detailed characterizations of this portion of the α -actinin molecule. Finally, we note that Barstead et al. (1991) have recently reported the sequence of *Caenorhabditis elegans* α -actinin. The nematode protein has an insertion of 27 residues, relative to previously sequenced α -actinins, in precisely the same location as does the *Drosophila* supercontractile muscle isoform. Although localized identically, the sequence of the *C. elegans* peptide is not similar to that of *Drosophila*, beyond having a high proline content (10 of 27 residues) suggesting that it is serving as a nonhelical spacer or hinge region of the protein.

It is surprising that the reduction of the nonmuscle α -actinin mRNA seen in *fliA*⁺ does not discernibly affect nonmuscle tissues. Both Schneider's cells and early embryos have

substantial amounts of this mRNA, and its virtual elimination would be expected to have a deleterious effect. Elimination of α -actinin in *Dictyostelium* by gene disruption did not confer a noticeable phenotype, suggesting that its function was largely redundant (Wallraff et al., 1986; Noegel and Witke, 1988; Schleicher et al., 1988). Our results are compatible with these observations, but do not clarify their significance. Assuming that the function of nonmuscle α -actinin is indeed largely redundant, two points should be emphasized. First, as stated above, we do not think that there are additional α -actinin genes in the *Drosophila* genome. Hence, any functional redundancy is almost certainly encoded by proteins whose primary sequences are unlike that of α -actinin. Second, although it is possible that *fliA*⁺ mutants synthesize small amounts of normal α -actinin, it is probably not responsible for the observed normal development of nonmuscle cells in these embryos. Several lethal alleles of the *Drosophila* α -actinin gene are presently being characterized in our laboratory. At least one of these has an inversion breakpoint within the transcribed region, after codon 38, and could not possibly encode functional protein (our unpublished results). This allele *lethal(1)2Cb⁺* (alias *l(1)EAS2^{Hc207}*), causes death well after the completion of embryogenesis, even when the maternal complement of α -actinin mRNA and protein is eliminated by creating germline clones homozygous for the mutation (refer to Perrimon et al., 1985). Our preliminary analyses of the cause of larval death suggest that it is due to progressive muscular paralysis accompanied by structural defects in muscles. Therefore, although warranting further direct investigation, most notably by analyses of somatic homozygous null clones, all of the available classical and molecular genetic evidence suggests that the function of α -actinin in *Drosophila* nonmuscle cells is largely or wholly redundant, and the selective value of this protein remains enigmatic. Our work does, however, make possible searches for interacting loci whose characterization may elucidate the role of nonmuscle α -actinin.

Why do all four *fliA* mutations paralyze indirect flight muscles but only weaken leg muscles? The trivial explanation is that flight muscle function is more sensitive to the observed structural perturbations than are leg and jump muscles. Unlike leg muscles, full activation of indirect flight muscles requires that they be alternately stretched by contractions of antagonistic fibers, and the efficient functioning of this oscillatory system critically depends on the mechanical resonance derived in part from myofibrillar elasticity (Pringle, 1978). All four mutations documented here probably reduce myofibril stiffness rather nonspecifically by perturbing their integrity. However, the principal effects of the mutations on flight muscles may be fairly specific, arising from their alterations of thin filament connectivity, or weakening of myofibrillar attachments to the sarcolemma (Fyrberg et al., 1990). Both of these effects may increase the elasticity of the fibers to the point where they cannot respond to stretch, and are hence paralyzed. These same alterations of elasticity would have a smaller effect on more conventional muscles, such as those of *Drosophila* legs. This hypothesis is testable. Flight and leg muscles of *fliA* homozygotes and heterozygotes mounted in a force transducer and subjected to rapid length changes (Jewell and Ruegg, 1966; Peckham et al., 1990) should exhibit significantly less resistance than that displayed by wild-type fibers.

That the single G>A transition in *fliA³* disrupts alternative splicing of *Drosophila* α -actinin mRNAs illustrates how profoundly exon sequences can influence utilization of adjacent donor sites. Our finding is most similar to that of Hodges and Rosenberg (1989), who reported that changing the last nucleotide of the fourth exon of the mouse ornithine transcarbamylase gene from G to A reduced usage of an adjacent donor site to 5% of its normal level and activated a cryptic splice donor site within the same intron. Whether these nucleotide alterations act by lowering the intrinsic strength of the splice donor sequence or by changing the physical properties of DNA in the exon-intron border is not known. However, we note that the wild-type sequence of the internal splice donor site (AG:GTTGGA) is a poor match for the eucaryotic consensus sequence (AG:GTGAGT) (Mount, 1982). It is possible that the G>A transition renders the donor site unrecognizable by the *Drosophila* splicing machinery.

The phenotypes of *fliA* mutants, and in particular those of *fliA¹* and *fliA⁴* flight muscles, suggest that α -actinin plays only a minor role in orchestrating the organization of thin filaments during myofibril formation, but has a principal role in stabilizing or anchoring them once localized within the sarcomere. We believe that these two alleles disrupt or reduce the accumulation of both muscle and nonmuscle α -actinin isoforms, and should hence perturb all phases of muscle development. However, myofibril assembly is surprisingly normal, and α -actinin therefore is unlikely to play a major role. Our only caveat regarding this theory is that it remains possible that the hypothetical orchestrating or organizing role is more redundant than the stabilizing role, and is accordingly more difficult to elicit using a genetic approach. However, the theory that α -actinin is primarily a thin filament stabilizer is consistent with roles proposed for its closest relatives, spectrin and dystrophin (see Byers et al., 1989, and Blanchard et al., 1989 for a discussion of sequence similarities). In the case of spectrin, immunofluorescent localization during *Drosophila* embryogenesis has shown that spectrin is recruited into regions defined by preexisting high concentrations of actin filaments, suggestive of a stabilizing, rather than organizational, role (Pesacreta et al., 1989). In the case of dystrophin, immunolocalization experiments and phenotypic analyses suggest that it is a cytoskeletal component that stabilizes the plasma membrane and associated protein (Koenig et al., 1988; Ervasti et al., 1990; Menke and Jockusch, 1991; Ervasti and Campbell, 1991), and that in its absence muscle fibers are more susceptible to work-induced injury (Stedman et al., 1991). Also, because muscle fibers form normally in Duchenne muscular dystrophy patients and subsequently degenerate (Cullen and Fulthorpe, 1975), dystrophin would seem more likely to have a stabilizing or anchoring function than an organizing role.

A final point for consideration concerns the basis for myofibrillar degeneration seen in *fliA³*. In theory, these effects could be caused by either a mechanical failure wherein weakened connections between thin filaments of adjacent sarcomeres caused them to break frequently, or by either local or global enhancement of necrotic activity within muscle fibers. Because both *fliA³* and *fliA⁴* mutants accumulate more or less equivalent low levels of α -actinin in flight muscles, degeneration is unlikely to result from mere reduction of α -actinin. Rather, atrophy must be triggered by a qualita-

tive difference between α -actinin synthesized in *fliA³* flight muscles and those synthesized within the same fibers in the other three *fliA* mutants. Further commentary on the specific cause of the observed degeneration must await more detailed histological studies, but it is remarkable that the most disruptive *fliA* mutant syndrome, that associated with *fliA³*, may be due to the expression of the inappropriate (supercontractile) isoform in flight muscles, rather than the accumulation of mutated α -actinin.

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