Molecular Biology of the Cell
Vol. 10, 135–149, January 1999

Differential Epitope Tagging of Actin in Transformed *Drosophila* Produces Distinct Effects on Myofibril Assembly and Function of the Indirect Flight Muscle

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Submitted June 26, 1998; Accepted October 28, 1998
Monitoring Editor: Tony Hunter

We have tested the impact of tags on the structure and function of indirect flight muscle (IFM)-specific Act88F actin by transforming mutant *Drosophila melanogaster*, which do not express endogenous actin in their IFMs, with tagged Act88F constructs. Epitope tagging is often the method of choice to monitor the fate of a protein when a specific antibody is not available. Studies addressing the functional significance of the closely related actin isoforms rely almost exclusively on tagged exogenous actin, because only few antibodies exist that can discriminate between isoforms. Thereby it is widely presumed that the tag does not significantly interfere with protein function. However, in most studies the tagged actin is expressed in a background of endogenous actin and, as a rule, represents only a minor fraction of the total actin. The Act88F gene encodes the only *Drosophila* actin isoform exclusively expressed in the highly ordered IFM. Null mutations in this gene do not affect viability, but phenotypic effects in transformants can be directly attributed to the transgene. Transgenic flies that express Act88F with either a 6x histidine tag or an 11-residue peptide derived from vesicular stomatitis virus G protein at the C terminus were flightless. Overall, the ultrastructure of the IFM resembled that of the Act88F null mutant, and only low amounts of C-terminally tagged actins were found. In contrast, expression of N-terminally tagged Act88F at amounts comparable with that of wild-type flies yielded fairly normal-looking myofibrils and partially reconstituted flight ability in the transformants. Our findings suggest that the N terminus of actin is less sensitive to modifications than the C terminus, because it can be tagged and still polymerize into functional thin filaments.

INTRODUCTION

Actins, a highly conserved family of cytoplasmic proteins, are among the most abundant proteins in eukaryotic cells. As a major component of the cytoskeleton, they control shape and motility in nonmuscle cells. In muscle, actin assembles into thin filaments, which together with interdigitating myosin thick filaments provide the framework for muscle contraction.

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Many organisms synthesize multiple isoforms of actin that are very similar in amino acid sequence even within the same cell. The differential expression of distinct actins as well as the high conservation of specific isoforms across species emphasize the functional importance of isoforms. In the case of actin, the question of how structure determines function appears to be particularly challenging. Considerable efforts have been made to understand how the different isoforms fulfill their various functions despite their extremely high sequence identity, and yet the basis of their functional diversity remains elusive.

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Studying the specific role of a particular actin isoform has always been hampered by the difficulty of discriminating between the introduced and the endogenous actins. Several experimental strategies have been designed to overcome this problem. For example, fluorescent labeling of actin was used to trace the fate of a distinct actin isoform after its microinjection into living cells (Sanger *et al.*, 1984), but this technique requires that significant amounts of actin be purified. Other labeling techniques such as the specific interaction of fluorescent phalloidin derivatives with filamentous actin (F-actin)[1] (Estes *et al.*, 1981; Collucio and Tilney, 1984) are dependent on the conformational state of the protein, because this toxin only binds to F-actin but not to monomeric actin. Alternatively, specific antibodies have been used to identify particular actins (Lubit and Schwartz, 1980). However, because actins are highly conserved, only a few isoform-specific antibodies devoid of cross-reactivity with homologous isoforms exist (Skalli *et al.*, 1986; Gimona *et al.*, 1994).

Epitope tagging has become a widely used approach of tracking different proteins with antibodies directed against the tag. A viral epitope such as the 11-amino acid peptide derived from vesicular stomatitis virus G protein (VSV-G) (Soldati and Perriard, 1991) decreases the risk that the antibody recognizing the tag cross-reacts with cellular components. Insertion of this particular tag at the C terminus of different actin isoforms has been used to study their distribution relative to the endogenous actins in fibroblasts and cardiomyocytes (von Arx *et al.*, 1995), smooth muscle, endothelial and epithelial cells (Mounier *et al.*, 1997), and hippocampal neurons (Kaech *et al.*, 1997). For the interpretation of these experiments it has been assumed that the tag does not interfere with the correct folding and function of the protein. Accordingly, heterologous muscle actins tagged at their C terminus with the 11-mer were found to coassemble with purified rabbit α-skeletal actin and did not perturb the sarcomeric organization when expressed in adult rat cardiomyocytes (von Arx *et al.*, 1995; Mounier *et al.*, 1997). However, in these experiments the large excess of unmodified endogenous actin is likely to overpower the properties of the modified recombinant actin. To rule out any dominant effects of unmodified endogenous over introduced actin, we have taken advantage of the indirect flight muscle (IFM) of *Drosophila melanogaster*, which allowed us to unambiguously analyze the consequences of epitope tagging the IFM-specific Act88F actin on muscle structure and function in its bona fide environment.

[1] Abbreviations used: F-actin, filamentous actin; 6xHis, 6x histidine; IFM, indirect flight muscle; UTR, untranslated region; VSV-G, vesicular stomatitis virus G protein.

Of the six actin genes in *Drosophila*, Act88F is expressed only in the IFM, where it is the sole actin isoform found (Fyrberg *et al.*, 1983; Ball *et al.*, 1987). Null mutations of the Act88F gene have yielded strains, for example, KM88 (Okamoto *et al.*, 1986), which because of the lack of endogenous Act88F actin in the IFM are flightless but otherwise perfectly viable. Valuable information on the significance of specific amino acids in myofibrillar assembly and function has been obtained from P element–mediated germ line transformation of such null mutants with mutated or truncated Act88F genes (Hiromi *et al.*, 1986; Reedy *et al.*, 1989; Drummond *et al.*, 1990, 1991). The different mutations produce a wide range of phenotypes, ranging from antimorphic effects (Karlik *et al.*, 1987; Sakai *et al.*, 1990) to a virtually normal IFM sarcomere organization (Drummond *et al.*, 1991).

Using this experimental system, we have examined the impact of epitope tagging on the structural and functional properties of the Act88F isoform *in situ* without the interference of endogenous normal actin. We have transformed Act88F null mutant KM88 flies lacking resident Act88F actin with Act88F constructs that bear either the 11-mer tag from VSV-G or a tag of six consecutive histidines (6xHis) at the C terminus or at the N terminus. Expression of the recombinant actin was demonstrated by means of the tag. Furthermore, by modifying either end of the molecule, we could examine how the position of the tag affects the processing, accumulation, and sarcomere assembly of tagged Act88F actin. The ultrastructural IFM morphology of N- and C-terminally tagged transformants was examined to assess the competence of tagged Act88F actin to polymerize and assemble into ordered myofibrillar structures. In parallel, by testing the flight ability of the corresponding transformants, we analyzed the consequences of epitope tagging Act88F actin on IFM function in vivo. Our studies demonstrate that addition of 6xHis at the N terminus does not abrogate the intrinsic property of actin to polymerize and therefore provides a valuable tool to isolate recombinant actin for in vitro studies.

MATERIALS AND METHODS

Plasmid Constructions

A *Pst*I–*Eco*RI fragment encoding the Act88F gene, 2-kb regulatory sequences, and the 3′ untranslated region (3′ UTR) was excised from the P[ry+;CSB] plasmid (Hiromi *et al.*, 1986) and cloned into the pW8 *Drosophila* transformation vector (Klemenz *et al.*, 1987), which contains the selectable white (w) marker gene (Figure 1A).

Insertion of a 6xHis Tag at the C Terminus of Act88F. A C-terminal 6xHis tag was introduced by PCR. Primers were designed to span the *Kpn*I site at the 5′ end (Act88F1, 5′-CGGCGGGTACCACCATGTACCCTGG-3′) and to generate six CAC codons, followed by a TAA translation stop signal, and an *Eco*RI site at the 3′ end (Act88F2, 5′-GCGAATTCTTAGTGGTGGTGGTGGTGGTGAAAGCATTTGCGGTGAACGATTCC-3′). Subsequently, the *Kpn*I–*Eco*RI

fragment of the original pW8Act88F construct (Figure 1A) was replaced by the *Kpn*I–*Eco*RI PCR product (Figure 1B).

Tagging Act88F with a C-terminal 11-Mer from VSV-G Protein. A VSV-G 11-mer tag (Soldati and Perriard, 1991) encoding the amino acids YTDIEMNRLGK was introduced at the C terminus of the Act88F coding sequence by PCR as described (Horton *et al.*, 1989) using two sets of primers: Act88F1 and 5′-CATCTCTATGTCTG-TATAAAAGCATTTGCGGTGAAC-3′, and 5′-GTTCACCGCAAA-TGCTTTTATACAGACATAGAGATG-3′ and 5′-GCGAATTCTTC-TTTCCTGCGTTATCCC-3′. The *Kpn*I–*Eco*RI fragment of the original pW8Act88F plasmid (Figure 1A) was replaced by the final PCR product (Figure 1C).

Deletion of 3′ UTR in pW8Act88F Plasmid. The *Kpn*I–*Eco*RI fragment in the original pW8Act88F plasmid (Figure 1A) was replaced by a DNA fragment amplified by PCR using the Act88F1 primer and an antisense primer (5′-CCGGAATTCTTAAAAGCATTTGCGGTG-3′) containing an *Eco*RI site following the TAA stop codon (Figure 1D).

Tagging Act88F with 6xHis and 11-Mer at the N Terminus. For inserting tags at the N terminus, the *Eco*RI site in the original pW8Act88F plasmid was obliterated, and instead a new *Eco*RI site was generated after the stop codon by PCR. Then, the nucleotide sequence at position −11 to −9 preceding the translation start codon was changed from 5′-CAA-3′ to 5′-GGC-3′ by site-directed mutagenesis to yield a unique *Stu*I site without affecting the splice acceptor sequence or the sequence immediately upstream of the start site. For cloning purposes, a *Bam*HI site (Gly-Ser) was inserted after the ATG by PCR. Finally, an oligonucleotide linker encoding the translational start site, followed by the 6xHis tag and an additional Ser (5′-TAGAAGGCCTGCCAAGATGCACCACCACCACC-ACCACTCCG-3′ and 5′-ATCCGGAGTGGTGGTGGTGGTGGTG-CATCTTGGCAGGCCTT-3′; Figure 1E) or the 11-mer tag with an additional Ser (5′-TAGAAGGCCTGCCAGGATGTACACCGA-CATCGAGATGAACCGCCTGGGCAAGTCCG-3′ and 5′-ATCC-GGACTTGCCCAGGCGGTTCATCTCGATGTCGGTGTACATCTT-GGCGGCGGCCTT-3′; Figure 1F), was inserted using the *Stu*I and *Bam*HI sites. The resulting constructs encoded the respective tag followed by a short sequence encoding a Ser-Gly-Ser tripeptide.

The correct reading frame of the modified Act88F genes was confirmed by DNA sequencing.

Germ Line Transformation

Germ line transformation was carried out essentially as described by Rubin and Spradling (1982) using the helper P element plasmid pπ25.7Δ2-3 wc. The recipient strain for all constructs was the KM88 mutant (w; Act88F^{KM88}) (Hiromi and Hotta, 1985; Okamoto *et al.*, 1986).

The posterior ends of homozygous KM88 embryos were injected with 100 ng/μl helper plasmid and 100–300 ng/μl pW8 constructs. Individual adult G_o flies were back-crossed to KM88 flies, and the progeny was scored for red eyes. For each construct at least four independent homozygous lines were established using balancer chromosomes (Lindsley and Zimm, 1992).

Flight Ability Test

Three days after eclosion, flies were tested for flight ability using the flight tester described by Okamoto *et al.* (1986). The flight tester consists of a transparent plastic cylinder that is 40 cm in diameter and 60 cm high. The bottom and the top are sealed with transparent plastic covers. A funnel with a 17-cm-long duct is inserted at the center of the top cover, and a saucer is hung 3 cm below the funnel. The cylinder is divided into intervals of 5 cm from bottom to top, the ceiling, the bottom, and the saucer. The inner surface is coated with liquid paraffin oil. The flight tester is illuminated from the top to attract flies. Flight ability is scored by releasing 200 flies through the funnel into the flight tester. After 3 min, the number of flies landing in each region was counted.

Electron Microscopy

IFMs were prepared for electron microscopy according to Reedy and Beall (1993) with minor modifications. Twenty-four- to 48-h-old female flies were etherized and mounted in modeling clay. The head and abdomen were removed, and the dorsal half of the thorax containing the dorsal longitudinal IFM was separated from the ventral half with microsurgical scissors. Dissected dorsal thoraces were directly immersed in a freshly prepared fixative consisting of 3% glutaraldehyde and 0.2% tannic acid in MOPS-buffered *Drosophila* Ringer's solution (Fyrberg *et al.*, 1990) without phosphate (pH 6.8) for 2 h at room temperature. After primary fixation, half-thoraces were rinsed three times for 15 min in MOPS-buffered *Drosophila* Ringer's solution and three times for 2 min in 100 mM phosphate buffer (pH 6.0). Subsequently, the thoraces were immersed for 1 h in ice-cold secondary fixative consisting of 1% osmium tetroxide in 100 mM phosphate buffer and 10 mM $MgCl_2$ (pH 6.0). After three washes in water for 5 min, thoraces were block stained in aqueous 2% uranyl acetate for 1 h at 4°C. After dehydration by a series of increasing ethanol concentrations (50–100%), specimens were embedded in a series of acetone-Epon mixtures and finally in pure Epon.

Electron micrographs were recorded on Kodak (Rochester, NY) SO163 film at a nominal magnification of 10,000× using a LEO (Oberkochen, Germany) 910 transmission electron microscope operated at 80 kV.

Protein Extraction

IFM Dissection. Individual adult flies were anesthetized with ether and mounted in a plasticine mold. The head and abdomen were removed, and a longitudinal incision was made along the dorsal surface of the thorax. The open thorax was then transferred into ice-cold *Drosophila* Ringer's solution and cut down the midsagittal plane, exposing the dorsal longitudinal flight muscles at the surface of each half-thorax. Using a fine needle, the IFMs were released from the thorax close to their site of attachment. The IFMs of one fly were transferred to 20 μl of SDS-PAGE sample loading buffer (2.3% SDS, 62.5 mM Tris-HCl, pH 7.0, 15% glycerol, 2.5% β-mercaptoethanol, 0.05% bromphenol blue) and boiled for 5 min.

PAGE and Immunoblot Analysis. Protein extracts corresponding to the IFMs of half a thorax were separated on 11.5% SDS-polyacrylamide gels (Laemmli, 1970). Gels were electroblotted onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were rinsed with PBS and transiently stained with Coomassie Brilliant Blue to confirm comparable amounts and equal transfer. After complete destaining with methanol, blots were washed in PBS and 0.1% Tween 20 and blocked for at least 30 min at room temperature in 5% milk powder in PBS and 0.1% Tween 20. Blots were incubated for 2 h at room temperature with a mAb recognizing different actin isoforms (Amersham Pharmacia Biotech, Zürich, Switzerland; diluted 1:7500 in PBS and 0.1% Tween 20), a mouse mAb recognizing the 6xHis tag (Dianova, Milan Analytica AG, La Roche, Switzerland; diluted 1:100) (Zentgraf *et al.*, 1995), or hybridoma supernatant from the mouse mAb P5D4, which recognizes the 11-mer tag from VSV-G (diluted 1:10) (Kreis, 1986). Blots were washed for 15 min each with 5% milk powder in PBS and 0.1% Tween 20, PBS and 0.1% Tween 20, and 1% blocking solution (Boehringer Mannheim, Rotkreuz, Switzerland) in PBS and 0.1% Tween 20, followed by a 2-h incubation with a 1:5000 dilution of a goat anti-mouse IgG alkaline phosphatase–conjugated secondary antibody (Sigma, Buchs, Switzerland). Blots were washed three times for 15 min with PBS and 0.1% Tween 20 and once with 100 mM Tris (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$, and then

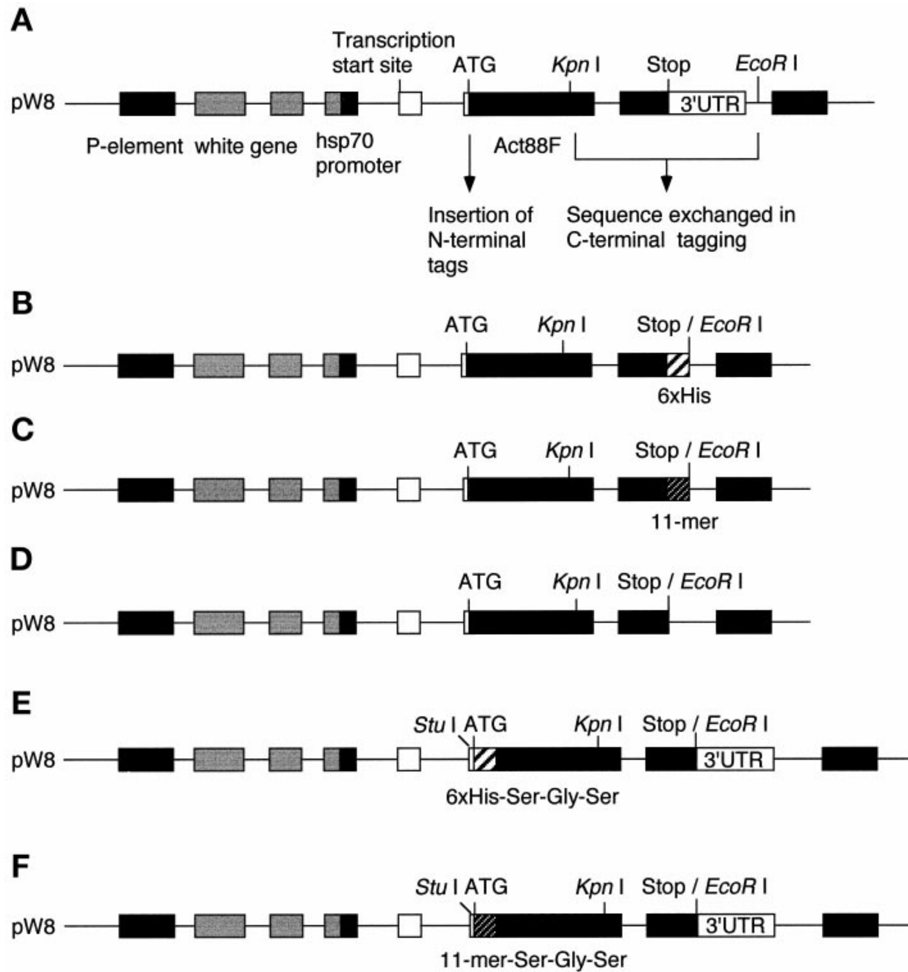

Figure 1. Schematic representation of tagged Act88F transformation constructs. The endogenous Act88F gene inserted into the pW8 vector (A) was modified at the C terminus by PCR and site-directed mutagenesis to contain a sequence corresponding to six consecutive histidines (B) or to an 11-mer derived from vesicular stomatitis virus G protein (C). This alteration resulted in the removal of the 3′ UTR. In the corresponding control construct, the 3′ UTR was removed from the endogenous Act88F (D). For tagging the Act88F at the N terminus, the sequences corresponding to six histidines or the 11-mer were introduced in the Act88F gene following the translation start site (E and F). Act88F coding sequences are represented by black boxes, whereas noncoding sequences are shown as white boxes. The epitope tags are shown as shaded boxes. Elements of the pW8 transformation vector are represented by stippled boxes.

developed with Western Blue stabilized substrate for alkaline phosphatase (Promega, Wallisellen, Switzerland).

RESULTS

Generation of Act88F6xHis and Act88F11-mer Transgenic Flies

To test the effect of epitope tags on the expression and function of actin, we modified the IFM-specific Act88F gene (Figure 1A), as described in MATERIALS AND METHODS, and introduced the recombinant actin into flies lacking endogenous Act88F expression. As shown schematically in Figure 1, B and C, sequences encoding 6 histidine residues or 11 amino acids derived from VSV-G protein (Soldati and Perriard, 1991) were inserted at the C terminus of the endogenous Act88F gene. The resulting pW8 transformation vectors were introduced into KM88, an Act88F null mutant line (Hiromi *et al*., 1986), by P element–mediated germ line transformation (Rubin and Spradling, 1982). Six Act88F6xHis and five Act88F11-mer homozygous fly lines were independently established. Two lines from each construct had insertions on the X chromosome, one and two had insertions on chromosome 2, and three and one had insertions on chromosome 3 for the Act88F6xHis and Act88F11-mer, respectively. Because addition of the tags at the C terminus of Act88F resulted in the removal of the 3′ UTR, five corresponding control lines were established, which are homozygous for Act88F lacking the 3′ UTR (Figure 1D). These control lines were all able to fly and exhibited an IFM structure that was indistinguishable from wild-type flies (our unpublished results). These findings indicate that the Act88F 3′ UTR is not required for the correct assembly of functional myofibrils.

In contrast to the C-terminal insertion of the epitope tags, addition of the 6xHis or the 11-mer tag at the N terminus (Figure 1, E and F) did not eliminate the 3′ UTR. For constructing N-terminally tagged transformation vectors, the tag sequences were inserted immediately after the ATG start codon in exon 2 of the Act88F

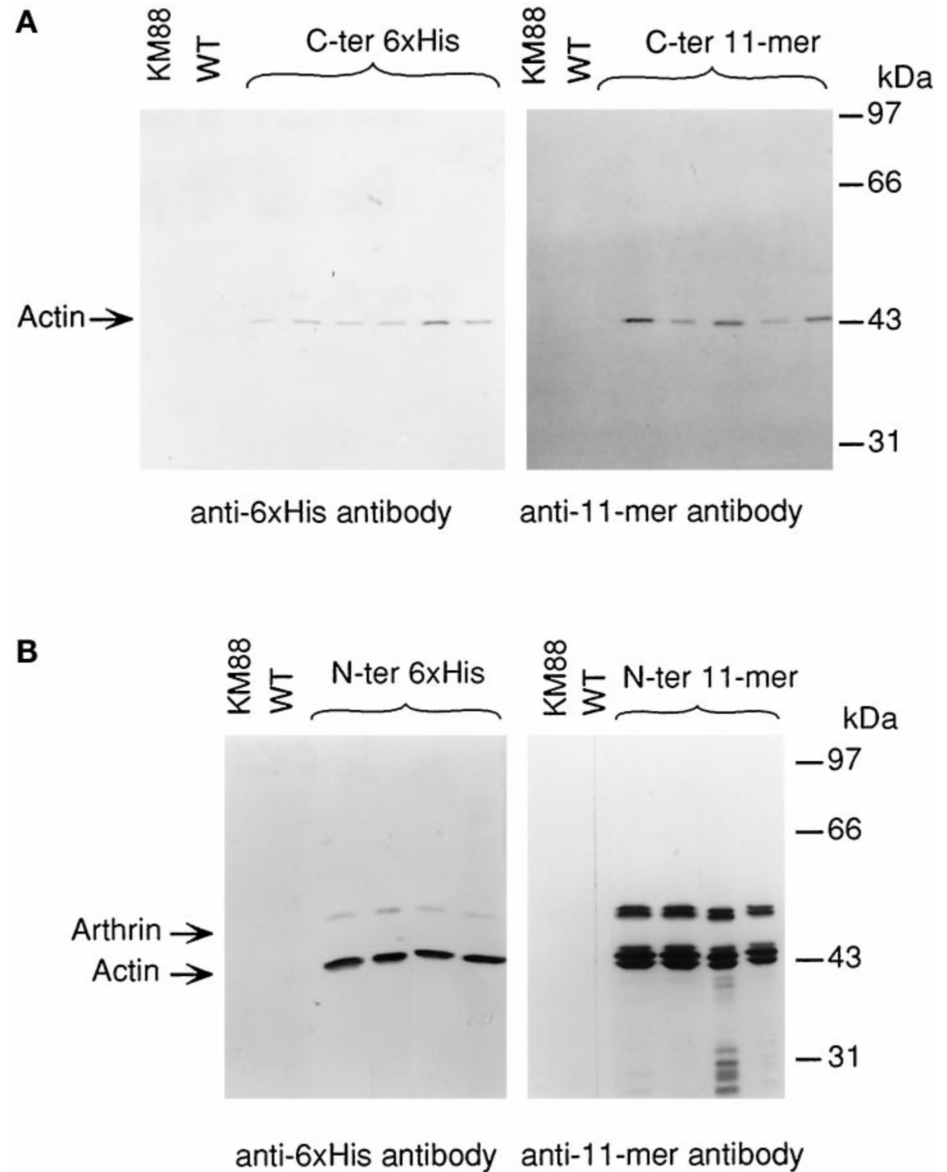

Figure 2. Expression of epitope-tagged Act88F in transformed KM88 flies. Protein extracts corresponding to an equal number of thoraces from transformants established with C-terminally (A) and N-terminally (B) tagged Act88F constructs were analyzed by immunoblotting. Wild-type (WT) and KM88 protein extracts were included on each blot as controls. Immunblots were incubated with either a mAb recognizing the 6xHis tag (left) or the 11-mer tag (right). (A) Each antibody recognizes a single band in extracts prepared from the transformed lines, which is absent in thoracic extracts from wild-type and KM88 flies. Its apparent molecular mass of 43 kDa corresponds to that of actin. Levels of tagged Act88F protein do not vary significantly among the transformants expressing the same construct. (B) In immunoblots from N-terminally tagged Act88F transformants, an additional band migrating at 55 kDa is detected. This band corresponds to tagged arthrin. In protein extracts from transformants tagged with the 11-mer at the N terminus, multiple actin and arthrin bands are present.

gene, followed by a Ser-Gly-Ser tripeptide linker. Four 6xHisAct88F and four 11-merAct88F homozygous fly lines with transgene integration on chromosomes 2 (one and three lines, respectively), 3 (one line each) or X (two 6xHisAct88F lines) were independently established.

Expression of Tagged Act88F Protein in IFM

The epitope tags were added to the coding sequence to allow for an unambiguous distinction of the recombinant Act88F from other endogenous isoforms in protein extracts of transformants by immunoblotting with mAbs that specifically recognize the respective tag. In Figure 2, the expression of C-terminally (Figure 2A) and N-terminally (Figure 2B) tagged Act88F in the IFM is shown. Each lane represents a protein extract of dissected IFM corresponding to one half thorax equivalent. Transient staining of the blots confirmed that total amounts of protein were comparable. The mAb against the 6xHis tag (Figure 2A, left) detected a single band, which migrates with an apparent molecular mass of ~43 kDa in all six transgenic lines. As expected, the band that corresponds to the size of actin is absent in the KM88 mutant and wild-type flies. Likewise, the P5D4 mAb recognizing the VSV-G 11-mer (Kreis, 1986) reacted with a single band of ~43 kDa in IFM extracts from flies transformed with the 11-mer Act88F construct (Figure 2A, right). C-terminally tagged Act88F protein was expressed in the IFMs of all

transgenic lines established with the corresponding construct. We did not observe any significant difference in the expressed levels of tagged Act88F between the individual lines from each construct.

Immunoblotting of IFM extracts from flies transformed with N-terminally tagged Act88F constructs with mAb against the respective tags showed that the constructs are expressed in the IFMs of the transformed flies at similar levels (Figure 2B). The mAb recognizing the 6xHis tag detected not only the prominent band representing tagged Act88F, but an additional tagged protein, which migrates with an apparent molecular mass of ~55 kDa (Figure 2B, left). Most likely, this band corresponds to 6xHis tagged arthrin, the ubiquitinated form of Act88F, which is typically present in insect IFM at the ratio of one arthrin molecule to six actin molecules (Ball *et al.*, 1987). The P5D4 mAb detected three bands with closely related molecular weights corresponding to actin and two bands representing tagged arthrin (Figure 2B, right). Because the epitope recognized by the P5D4 antibody predominantly consists of the five C-terminal amino acids of the 11-mer (Kreis, 1986) it is possible that partial removal of the N-terminal amino acids accounts for the multiple actin forms. Alternatively, the N-terminal 11-mer tag leads to inefficient posttranslational processing. Whereas the exact origin of the multiple bands is unclear, it appears that the 11-mer tag interferes with the correct processing of the N terminus without, however, abrogating ubiquitination.

Immunoblots using the corresponding antibodies revealed an increased amount of the tagged actin in IFM extracts from transformants expressing N-terminally tagged actins compared with the immunoblots of IFM extracts from C-terminally tagged Act88F lines (Figure 2, compare A and B). Together with the detection of tagged arthrin, this finding suggests that N-terminally tagged Act88F is present at higher levels than the C-terminally tagged Act88F.

Effects of Tagged Act88F Expression on Flight Ability

Because the IFM-specific Act88F is absent in the KM88 null mutant, a viable but flightless line ensues (Hiromi and Hotta, 1985; Okamoto *et al.*, 1986). The sarcomeric organization and the myofibrillar structure, as well as the flight ability of transformants expressing the unmodified Act88F gene, were shown to be indistinguishable from wild-type flies (Hiromi *et al.*, 1986; our unpublished results). We tested the effects of the epitope tags on the functional properties of Act88F in a flight test assay (Okamoto *et al.*, 1986; see MATERIALS AND METHODS). Figure 3 displays histograms characterizing the flight ability of the transformants compared with that of wild-type flies and the KM88 null mutant, respectively. Several lines were analyzed for each type of transformant. The results for the different lines were comparable, and a representative histogram is given for each type of transformant. The histograms of the C-terminally tagged transformants (Figure 3, D and F) looked similar to the histogram of the flightless KM88 mutant (Figure 3B). Accordingly, the majority of the flies stayed in the saucer or fell to the bottom of the cylinder. A few flies reached the sidewall of the cylinder near the bottom, probably because of the random trajectories of their falls. The lack of flight ability in both C-terminally tagged transformants indicates that insertion of either six histidines or the 11-mer epitope at the C terminus of Act88F interferes with the assembly and/or function of thin filaments.

Normal flight ability is defined by >50% of the flies reaching the sidewall in the top third of the cylinder, which corresponds to the height of the saucer and higher. Accordingly, the histogram for wild-type flies (Figure 3A) reveals that 64.4% reach the top third. Approximately 30% of the homozygous transformants expressing Act88F with an 11-mer epitope at the N terminus (Figure 3E) and 13% of the lines with an N-terminally 6xHis-tagged Act88F (Figure 3C) were able to land in the top third. However, 35.5% of the 11-mer flies and 51.5% of the 6xHis flies reached the sidewall at a height between 5 and 40 cm. These results suggest that the expression of N-terminally tagged Act88F, although not normal, reconstitutes IFM function to a significant extent.

Ultrastructural Analysis of the IFM

We have used transmission electron microscopy of embedded and thin-sectioned specimens to examine the consequences of epitope tagging on the IFM organization and morphology. For this purpose, the IFMs of adult flies (24–48 h after eclosion) were fixed *in situ* and embedded and sectioned as described in MATERIALS AND METHODS. Figures 4 and 5 display representative electron micrographs of longitudinal (Figure 4) and transverse (Figure 5) sections of the IFMs from C- and N-terminally tagged transformants in comparison with wild-type flies. In contrast to the well-organized sarcomeres of wild-type flies (Figure 4A), sarcomeric organization is virtually absent on longitudinal sections of C-terminally tagged Act88F flies (Figure 4B). Indications of lateral alignment of thick filaments may still occur in places. The randomly distributed thick filaments and the apparent lack of thin filaments and Z discs are reminiscent of the appearance of the KM88 null mutant IFM (Beall *et al.*, 1989; our unpublished results). In contrast, in longitudinal sections the N-terminally tagged (i.e., with 6xHis or 11-mer) transformants (Figure 4, C and D) display a sarcomeric organization similar to that of wild-type flies. In the transformants expressing 6xHis Act88F

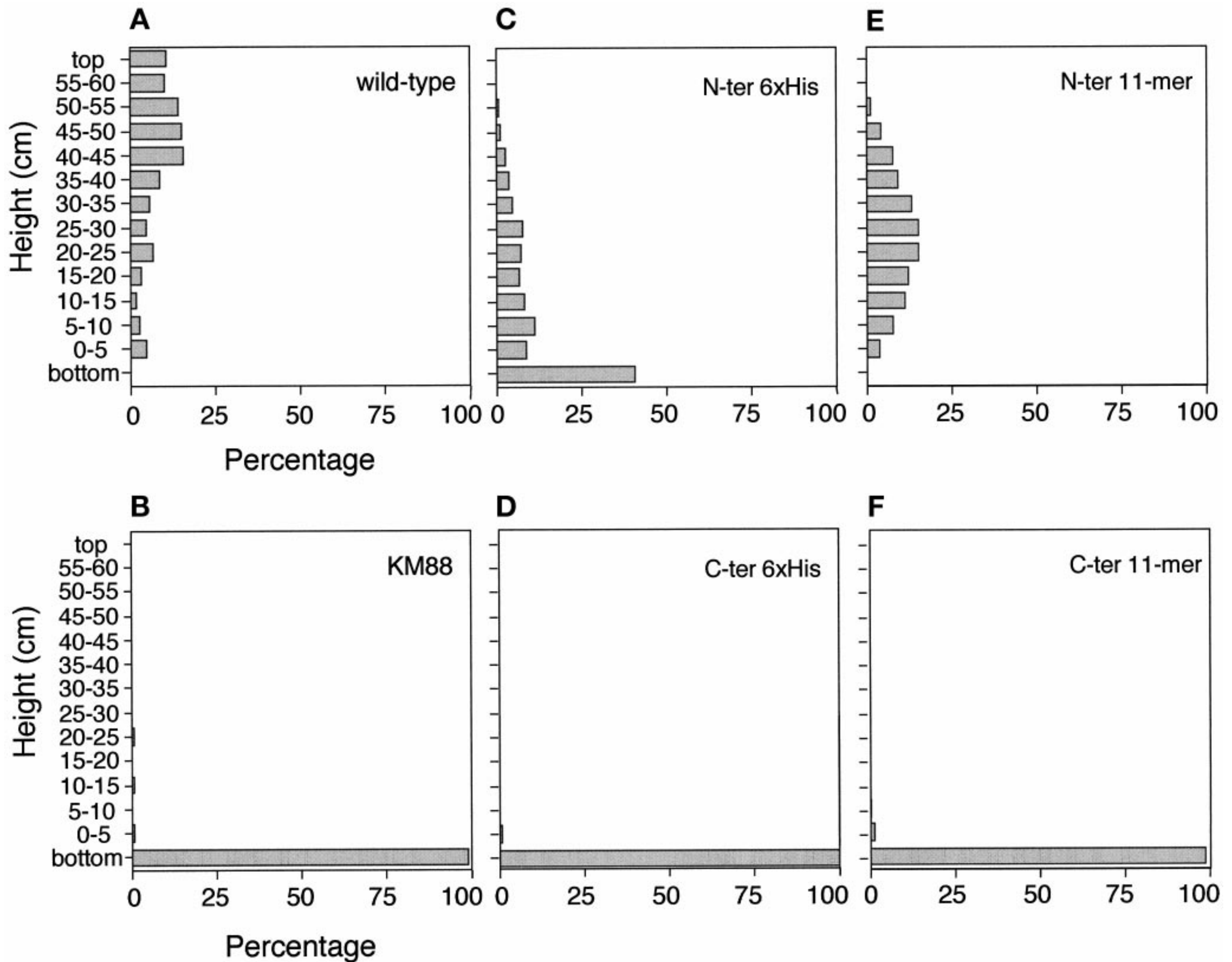

Figure 3. Effects of epitope tagging on IFM function in transformed *Drosophila*. Flight ability was examined in a flight tester (Okamoto *et al.*, 1986). Accordingly, the "landing sites" of individual flies were scored and are shown as percentages. A consistent fraction of flies (approximately one-third) was found either at the bottom of the cylinder or remained in the saucer and was subtracted from the nonflying flies from each test. Each histogram represents the average of three experiments. (A) Wild-type flies show heterogeneity in their flight ability. (B) The KM88 null mutant is flightless. (C) In N-terminally tagged 6xHis transformants, the percentage of poor fliers is higher than in wild-type flies. (D) C-terminally tagged 6xHis transformants are not able to fly. (E) Transformants with the 11-mer tag at the N terminus have a slightly increased flight ability compared with flies with a 6xHis tag, but they do not fly as proficiently as wild-type flies. (F) Similar to the C-terminally tagged 6xHis transformants, C-terminally tagged 11-mer transformants are flightless.

(Figure 4C) as well as in those expressing 11-mer Act88F (Figure 4D), thin filaments alternating with thick filaments are evident. However, subtle morphological defects are depicted in the 11-mer Act88F transformants (Figure 4D). Along the periphery of the myofibrils, fraying of thick filaments (Figure 4D, arrows) is often discernible. The imperfections in the lateral register of thick filaments suggest the absence of thin filaments in these peripheral myofibril regions.

Cross-sections of wild-type flies (Figure 5A) and N-terminally tagged transformants (Figure 5, C and D) typically reveal myofibrils that are round and rather uniform in diameter. The myofibrils from flies with 6xHis-tagged Act88F (Figure 5C) appear slightly smaller (~10%) in diameter than those of wild-type IFM and N-terminally 11-mer–tagged Act88F transformants. Just as with wild-type flies, the thin and thick filaments of both N-terminally tagged transformants are arranged in a highly regular hexagonal array. However, the myofibrils from flies expressing the 11-mer–tagged Act88F exhibit occasional disturbances in the hexagonal packing of thin and thick filaments (Figure 5D, white arrows and inset). These disturbances are clearly distinct from the sporadic missing

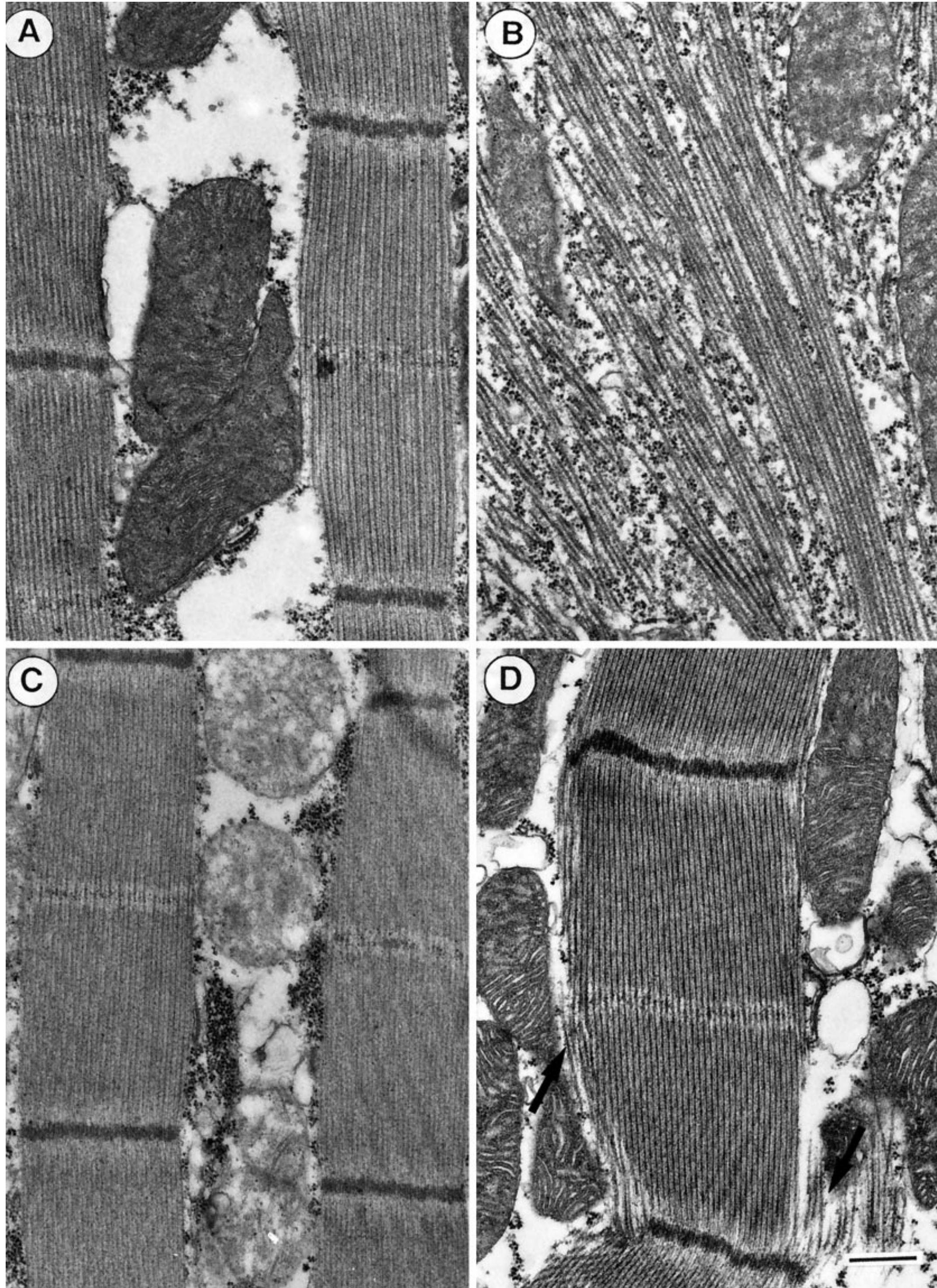

Figure 4. Sarcomeric organization and myofibril morphology are preserved in N-terminally tagged transformants. (A) Electron micrograph of a longitudinal section of wild-type IFM showing the highly ordered sarcomeric organization with alternating thick and thin filaments, Z discs, and central M lines. (B) Longitudinal sections of C-terminally tagged transformants reveal only thick filaments that are randomly distributed. There is no apparent assembly of thin filaments. (C) IFM of N-terminally tagged 6xHis transformants displays myofibrils similar to those of wild-type IFM, with well-defined sarcomeric structures in which M lines and Z discs are clearly discernible. (D) In sarcomeres of N-terminally tagged 11-mer transformants, the register of alternating thick and thin filaments becomes slightly perturbed at the periphery (black arrows). Bar, 500 nm.

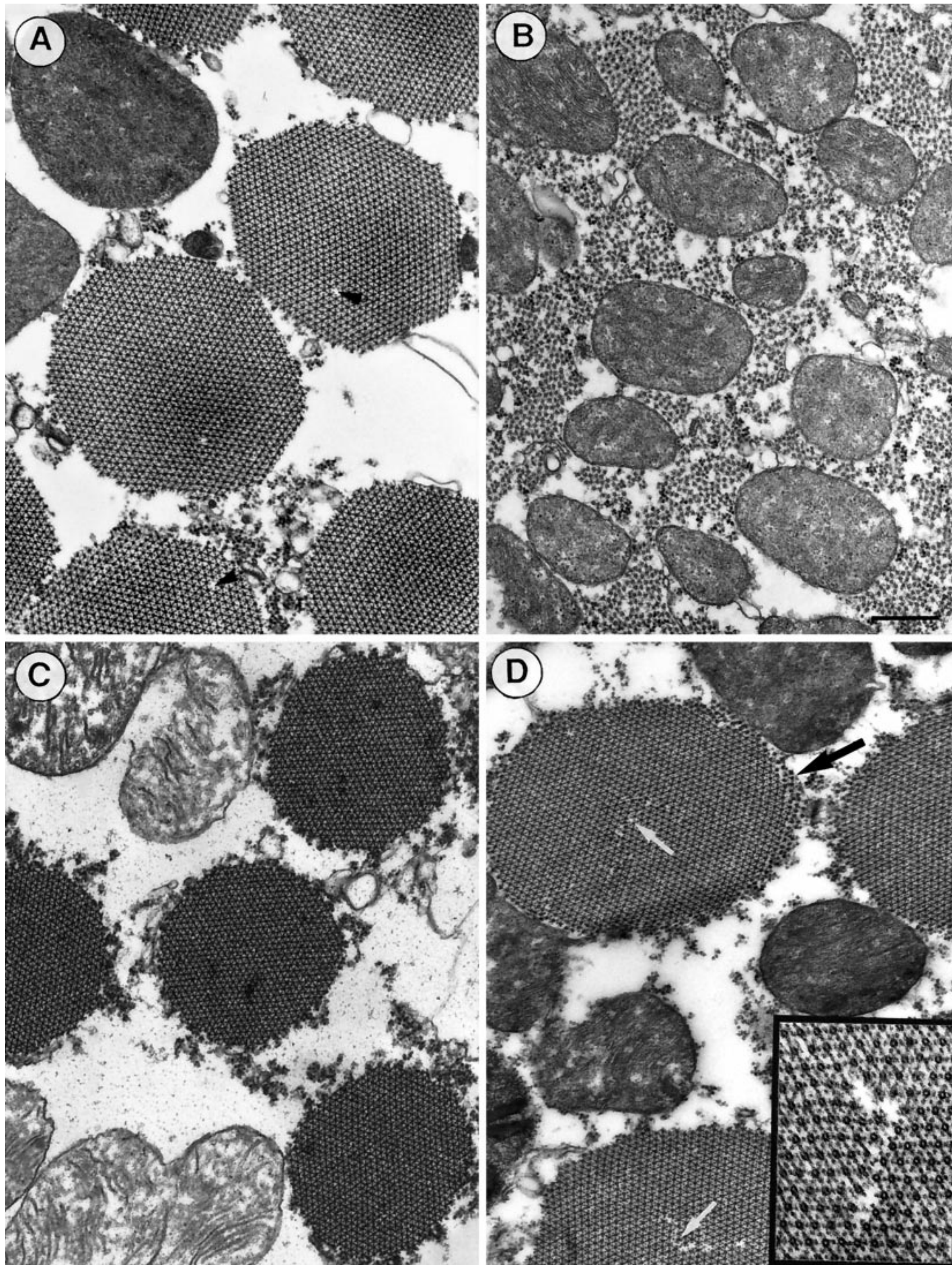

Figure 5. Comparison of transverse sections from IFMs of wild-type and transformed flies. (A) In the wild-type IFM, myofibrils are round, uniform in diameter, and well ordered. They form hexagonal arrays of one thick myosin filament surrounded by six thin actin filaments. As marked by the black arrowheads, a thick filament is occasionally missing in an otherwise undisturbed filament lattice. (B) In transverse sections of C-terminally tagged transformants, there are no myofibrils assembled. As in cross-sections of the KM88 null mutant, thick filaments appear to be more or less randomly distributed and thin filaments are not evident. (C and D) Ordered myofibrillar structures are present in N-terminally tagged transformants. However, subtle effects of the tag on the structure are revealed. (D) In transformants expressing N-terminally 11-mer–tagged Act88F, the edges of the myofibrils appear frayed (black arrow). Occasionally, disturbances are detected in the hexagonal lattice of thick and thin filaments (white arrows). The inset reveals one of these disturbances at a twofold higher magnification. (C) On average, the myofibrils of transformants with an N-terminal 6xHis tag appear to have a slightly smaller (~10%) diameter than myofibrils of wild-type IFM. Bar, 500 nm.

of a thick filament in an otherwise undisturbed hexogonal myofilament lattice of wild-type flies (Figure 5A, black arrowheads; Sparrow *et al.*, 1991). Moreover, lattice discontinuities at the periphery (Figure 5D, black arrows) are consistent with the fraying of thick filaments along the edge of myofibrils observed in longitudinal sections (Figure 4D, arrows). In contrast, no thin filaments or hexagonal packing of thick filaments were observed in cross-sections of the C-terminally tagged Act88F transformants, just randomly distributed thick filaments with no clear delineation into distinct myofibrils (Figure 5B).

In flies expressing C-terminally tagged Act88F, the complete absence of myofibril morphology, from the absence of thin filaments to the absence of sarcomeric organization, accounts for the flightless phenotype observed for the transformants. In contrast, IFMs of N-terminally tagged transformants exhibit a largely normal muscle morphology with well-organized sarcomeres and highly ordered myofibrils comprising thin and thick filaments. As a result, these transformants are able to fly, albeit slightly less efficiently than wild-type flies. It is conceivable that the morphological imperfections observed in the IFMs of N-terminally tagged transformants (as described above) are responsible for the reduction in flight ability.

The Position of the Tag Influences the Amount of Act88F Present in the IFM

To test whether the failure to form proper myofibrils in the C-terminally tagged Act88F transformants was correlated with reduced amounts of recombinant actin in the IFM, we compared the amounts of tagged Act88F in the IFMs of transformants with the amounts of endogenous Act88F in wild-type flies. For this purpose, protein extracts of the null mutant KM88, wild-type, and transgenic flies were analyzed by immunoblotting with a monoclonal anti-actin antibody that recognizes most actin isoforms (see MATERIALS AND METHODS). As documented in Figure 6, the blot probed with this antibody, which equally recognized endogenous Act88F in wild-type flies as well as tagged Act88F in transformants, revealed C-terminally tagged transformants to accumulate significantly less actin in their IFM than wild-type flies. In addition, the ubiquitinated actin species that migrates as a 55-kDa band in IFM extracts from wild-type flies is not detectable in IFM extracts from C-terminally tagged transformants. Because only every seventh Act88F molecule is ubiquitinated in wild-type flies (Ball *et al.*, 1987), the amount of tagged arthrin in IFM extracts from C-terminally tagged transformants may well be below the limits of detection. However, overloading gels with IFM extracts to yield amounts of tagged Act88F equivalent to the amount of wild-type Act88F where arthrin is detectable did not result in the detection of tagged arthrin in the transformants (our unpublished results). Alternatively, the tag at the C terminus might interfere with the ubiquitination process.

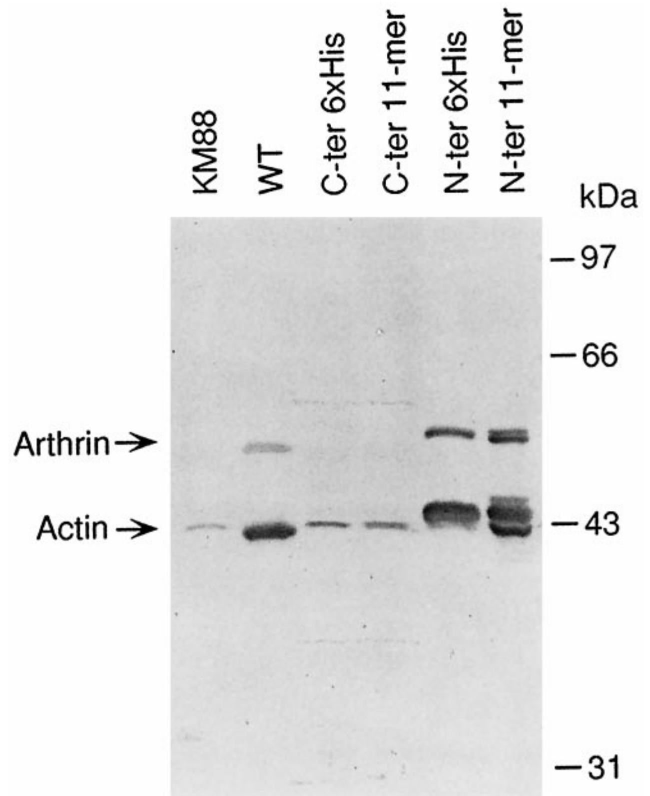

Figure 6. The position of the tag affects the level of Act88F expression. Immunoblotting of IFM extracts from KM88 null mutants, from wild-type flies, and from tagged Act88F transformants with a monoclonal antibody that recognizes different actin isoforms reveals that the amounts of C-terminally tagged actin are significantly lower than the amounts of Act88F in wild-type (WT) flies. Act88F with an N-terminal tag is expressed at levels similar to endogenous Act88F in wild-type IFM. N-terminally tagged arthrin (~55 kDa) is also detected by the anti-actin antibody. Consistent with the immunoblot shown in Figure 2, the antibody recognizes multiple 11-mer–tagged Act88F and arthrin bands. The band in the IFM extract of KM88 represents non-IFM actin isoforms.

In contrast, the expression levels of the N-terminally tagged Act88F are similar to wild-type Act88F levels, and ubiquitin conjugation occurs in 6xHis– as well as 11-mer–tagged Act88F transformants. In the latter, the monoclonal anti-actin antibody recognized multiple bands, which correspond to the three tagged actin and the two tagged arthrin bands detected by the P5D4 mAb to the 11-mer epitope (Figure 2B, right). This result confirms that the individual bands represent variants of 11-mer–tagged recombinant actin. Somewhat unexpected, small amounts of actin (~5% of the wild-type amount) were also detected in the KM88 null mutant. It has been suggested that cytoplasmic actin is a minor actin species in the IFM (Fyrberg *et al.*,

1983). Although the band detected in extracts from KM88 could represent cytoplasmic actin from IFM, we believe it rather represents other *Drosophila* actin isoforms from surrounding muscle or nonmuscle tissue, especially because in the absence of a discernible myofibrillar structure in KM88, it is extremely difficult to exclusively dissect IFM.

DISCUSSION

In C-terminally Tagged Transformants Functional Thin Filaments Are Not Detectable

The low amount of C-terminally tagged Act88F present in the IFMs of corresponding transformants suggests that the thus modified actin is either not properly folded and/or cannot polmerize into actin-containing thin filaments, thereby becoming susceptible to degradation. The inability of actin to assemble into thin filaments, in turn, leads to the absence of sarcomeric organization and, ultimately, to the loss of flight ability. These results indicate that the actin conformation and/or polymerization is sensitive to certain modifications of its C terminus. A number of experiments involving deletions or mutations of the C-terminal sequence have demonstrated the importance of this region for proper F-actin polymerization and stability. For example, mutating the conserved cysteine at position 374 to a serine in the chicken β-cytoplasmic actin increased the critical concentration for polymerization by more than fivefold (Aspenström *et al.*, 1993). Removal of either the two or three C-terminal residues of actin resulted in actin filaments with increased fragility and flexibility (O'Donoghue *et al.*, 1992; Mossakowska *et al.*, 1993).

Interestingly, other modifications of the C terminus such as labeling of Cys-374 with fluorescent probes (Kouyama and Mihashi, 1981; Cooper *et al.*, 1983; for review see Miki *et al.*, 1992) or undecagold (Milligan *et al.*, 1990) do not significantly interfere with the polymerization characteristics of actin or the filament structure as predicted by the Holmes model (Holmes *et al.*, 1990; Lorenz *et al.*, 1993). Likewise, it has been reported that addition of the VSV-G 11-mer epitope at the C terminus of muscle as well as nonmuscle actin isoforms did not impede their in vitro copolymerization with rabbit skeletal muscle actin (von Arx *et al.*, 1995; Mounier *et al.*, 1997). After transfection, coassembly or association of recombinantly expressed tagged actin isoforms with the preexisting microfilament system was observed for a variety of cell types. It should be noted, however, that recombinant actin usually amounts to <10% of the total actin in transfected cells. Thus, it is conceivable that the excessive amounts of endogenous actin mask the effective properties of the less abundant recombinant actin.

The Heidelberg model of the F-actin filament, which has evolved from the atomic structure of the monomeric actin–DNaseI complex (Kabsch *et al.*, 1990), in combination with x-ray fiber diffraction data (Holmes *et al.*, 1990; Lorenz *et al.*, 1993) appears largely consistent with the extensive biochemical data on actin at hand. However, a significantly different model has been proposed by Schutt *et al.* (1995a,b, 1997) underscoring that the ultimate structure of the actin filament at atomic scale has remained elusive. In particular, there are some uncertainties regarding the highly mobile N terminus and the C-terminal helix (residues 368–375). Because the definite structure in the filament of precisely those regions of the molecule that are modified by the tags is unknown, predictions on the structural consequences of the tag are subject to speculation. In fact, only in rare instances have the consequences on filament structure caused by the various mutations in actin been analyzed (Orlova *et al.*, 1997). Nevertheless, based on the predicted location of the C terminus in the vicinity of the interface between the two long-pitch helical strands, it appears reasonable to assume that modifications in this region somehow affect subunit–subunit interactions. Both the 6xHis and the 11-mer tags are significantly larger than pyrene, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphtyl)ethylenediamine or even undecagold and, therefore, in contrast to these modifications, may interfere with the proper interaction of the two long-pitch helical strands of the F-actin filament so that no stable filaments are formed, and then, in an in vivo environment, the tagged actin is rapidly degraded. The low levels of C-terminally tagged Act88F observed in our transformants are consistent with this hypothesis. According to the Holmes model of the F-actin filament, there is sufficient space at the C terminus in the Holmes model to sterically accommodate the 6 histidines or the 11 amino acids of the 11-mer tag without causing a major structural change of the filament. However, it has been shown that conformational coupling of C-terminal modifications with more distant sites does exist for both the monomer and polymer forms of actin (Drummond *et al.*, 1992; Crosbie *et al.*, 1994; Moraczewska *et al.*, 1996). Hence, addition of the tags to the C terminus of Act88F might affect the F-actin structure and/or conformation. Such structural changes might have an effect on the interaction of the C terminus with actin-binding proteins so that ultimately the function of the F-actin is modified.

Ubiquitination of C-terminally Tagged Act88F Actin Does Not Occur in Homozygous Transformants

The apparent absence of ubiquitinated actin in C-terminally tagged homozygotes (see Figures 2A and 6) suggested that the modification of the C terminus interfered with IFM-specific ubiquitination. However, in heterozygotes with one copy of the C-terminally tagged Act88F gene and one wild-type Act88F gene,

we have observed thin filaments in the IFM. In extracts of these IFMs, a ubiquitin conjugate was detected by antibodies recognizing the tag (our unpublished data), indicating that interference of the C-terminal tag with ubiquitination is an unlikely explanation for the absence of tagged arthrin in homozygous transformants. Ubiquitination lags several hours behind Act88F expression and thus parallels myofibril formation (Ball *et al.*, 1987). It is conceivable that actin ubiquitination might require or be regulated by thin filament formation. Our findings support this notion, because in the IFM of homozygous transformants, C-terminally tagged Act88F is unable to form thin filaments, and hence, ubiquitination does not occur.

Tagging the N Terminus of Act88F Does Not Significantly Interfere with Myofibrillar Structure and Function

Evidently, the amino acid sequence at the N terminus of actin is crucial for its structural and functional integrity (Cook *et al.*, 1991, 1992; Reedy *et al.*, 1991; Aspenström *et al.*, 1992; Miller *et al.*, 1996). Thus, it was surprising that the N-terminal fusion of the relatively large green fluorescent protein to *Dictyostelium* actin did not prevent the hybrid protein from copolymerizing with endogenous actin (Westphal *et al.*, 1997). Likewise, transformants exclusively expressing N-terminally tagged Act88F in their IFMs exhibit a virtually normal sarcomeric organization with alternating thin and thick filaments and are able to fly, although not as proficiently as wild-type flies. These results demonstrate that N-terminally tagged actin is by itself polymerization competent, and that the resulting thin filaments are at least partially functional in a flight test.

Consistent with the compromised function of the corresponding IFM, subtle effects on the morphological phenotype became apparent at the ultrastructural level. In IFMs of 11-mer–tagged Act88F flies, occasional disruptions of the hexagonal myofibril array were observed. A similar phenotype has been described in transformants with the single point mutation E316K in Act88F (Drummond *et al.*, 1990). Unlike the N-terminal tags, this mutation involving a glutamic acid to lysine change at position 316 abolished flight ability. More specifically, it was found to alter cross-bridge kinetics, although it is distant from the nearest known myosin or tropomyosin contact. As discussed above in the context of the C-terminal modifications, this mutation may be affecting the interaction of actin with myosin through a long-range conformational change within the F-actin polymer. Moreover, in *Drosophila* expressing mutated myosin, defects in the actomyosin cross-bridges have been described to account for disruptions of the hexagonal packing of thin and thick filaments in the IFM (Mogami and Hotta, 1981; Warmke *et al.*, 1992).

Tags at the N Terminus of Actin May Influence Actin–Myosin Interactions

Over the past few years, significant progress has been made on mapping the binding sites on the F-actin filament of a number of actin-binding proteins through the combination of maps obtained by electron microscopy with atomic structures determined by x-ray diffraction and structural nuclear magnetic resonance (for review see McGough, 1998, and references therein). These studies emphasized the importance of the filament geometry (i.e., the packing of actin subunits in the helix) and of conformational flexibility in defining the molecular interactions between actin and actin-binding proteins (Chik *et al.*, 1996; McGough *et al.*, 1997). In most cases, actin subdomain 1, where both the N- and C termini reside, contributed to the respective binding site. At the C terminus, residues from ~340 to 355 appear to be involved in a number of binding sites (Rayment *et al.*, 1993; McGough *et al.*, 1994, 1997; Schmid *et al.*, 1994; Hanein *et al.*, 1997). In light of these findings, the absence of thin filaments in the IFMs of C-terminally tagged transformants may be explained by conformational changes brought about by the tags such that interactions with actin-binding proteins, which are relevant for myofibril assembly in the IFM, are altered or can no longer occur. Alternatively, the tags may reduce the mobility of the C terminus, which is required to substantially move during filament formation (Milligan, 1996).

Proper actin–myosin interactions are thought to be important for the correct registration of thick and thin filaments within the sarcomere (Reedy and Beall, 1993). If so, alterations in cross-bridge formation attributable to actin or myosin mutations would be expected to cause aberrant myofibrillar assembly. At the N terminus of rabbit skeletal actin, the six negatively charged amino acids, Asp^1, Glu^2, Asp^3, Glu^4, Asp^{24}, and Asp^{25}, are getting in close contact with the myosin loop Tyr^{626} to Gln^{647} during the cross-bridge cycle and are believed to be predominantly involved in ionic interactions with myosin beside the primary myosin binding site involving the helix-loop-helix near the C terminus of actin (Ile^{341} to Gln^{354}), a loop between Ala^{144} and Thr^{148} on the same actin monomer, and part of the DNase I binding loop (His^{40}-Gly^{42}) on an adjacent actin monomer (for review, see Milligan, 1996). Modification of the charge environment at the N terminus by either tag could affect these ionic interactions and interfere with the normal cross-bridge formation. Moreover, it has been shown that mutations in the N-terminal region of actin, which yielded a change of charge, affect myosin S1 binding to actin and thereby reduce in vitro motility (Aspenström and Karlsson, 1991; Aspenström *et al.*, 1992; Sutoh *et al.*, 1991; Miller *et al.*, 1996). However, N-terminal mutations that do not induce charge changes may

also affect actomyosin interactions. For example, in the Act88F^{G6AA7T} double mutant, peripheral thin and thick filaments are out of register. Moreover, the unincorporated thin filaments at the periphery point in the opposite direction, as indicated by the reversed orientation of myosin chevrons (Reedy *et al.*, 1989). Like the N-terminally tagged Act88F transformants, these mutants are flightless despite a core of precisely interdigitated thin and thick filaments.

It is conceivable that the extra charge introduced by the six histidines and/or the structural changes arising from the additional residues in the N-terminally tagged transformants also affect the actomyosin interactions. The unincorporated thick filaments at the periphery of the myofibrils support this hypothesis. However, these rather minor ultrastructural defects and the flight ability in particular argue against a severe effect of these two N-terminal tags on the actomyosin interaction. Future experiments should provide insight into the structural details of the actomyosin rigor complex.

Alternatively, the thin–thick filament lattice disturbances observed in the IFM (see Figures 4 and 5) could result from an imbalance of the actin-to-myosin stoichiometry. Consistent with an imbalance between thin and thick filaments (Beall *et al.*, 1989; Cripps *et al.*, 1994), we have observed fraying of their hexagonal packing with unintegrated thick filaments only residing at the periphery of myofibrils.

The accumulation of N- and C-terminally tagged Act88F in the IFM differs drastically. Comparison of the respective phenotypes strongly suggests that the amount of actin plays an important role in myofibril assembly and/or organization. Studies on Act88F mutations that yield a reduced amount of actin over myosin support the notion that reduced accumulation of actin in the IFM might produce structural and functional myofibrillar defects. For example, in the point mutant V339I (Drummond *et al.*, 1991), the monomeric actin conformation appears largely unaltered; nevertheless, the mutant flies display a very disrupted IFM structure and functionally are flightless, a phenotype that has been related to reduced amounts of the mutant actin relative to myosin. A number of experiments provide evidence that an imbalance of the ratio between thin and thick filaments rather than the absolute deficit of one of these component appears to be responsible for the myofibrillar defects observed (Beall *et al.*, 1989). Hence, it is conceivable that the subtle morphological defects observed in the N-terminally tagged Act88F transformants are possibly due to a small imbalance of tagged Act88F over myosin. Although immunoblot experiments indicate that expression of N-terminally tagged Act88F and wild-type Act88F is similar, small variations in expression that go undetected by this technique might nevertheless have definite structural and functional consequences.

Several myofibrillar protein heterozygous null mutants with only one copy of the normal gene exhibit out-of-register myofilaments at the periphery of the myofibrils, similar to those seen in the homozygous N-terminally tagged Act88F transformants. For example, heterozygotes for the KM88 null mutation, which are flightless, display myofibrils with a core of hexagonally packed thin and thick filaments surrounded by unintegrated thick filaments (Beall *et al.*, 1989). We observed a corresponding phenotype in heterozygotes with one wild-type and one C-terminally tagged Act88F gene (our unpublished results). The core of hexagonally packed thin and thick filaments could be conceived as myofibril with a smaller diameter. For comparison, myofibrils of transformants homozygous for 6xHis-tagged Act88F also had a slightly smaller diameter than the IFM myofibrils of wild-type flies but were still able to fly.

The IFM provides a unique experimental system to assay both qualitatively and quantitatively the effects of modifying Act88F actin. In the absence of endogenous protein, specifically tagged Act88F actin can be tested at increasing levels of stringency ranging from specific protein accumulation to quantitative assessment of rescuing flight. Further analysis of the consequences of these tags on actin polymerization and filament structure will require physicochemical studies with purified proteins. Such experiments are now feasible with the use of affinity purification procedures based on the metal-binding properties of the polyhistidine tags.

ACKNOWLEDGMENTS

We thank Professor Jean-Claude Perriard (Swiss Federal Institute of Technology, Zürich, Switzerland) for providing the α-cardiac actin 11-mer construct. The P5D4 antibody was a kind gift from the late Professor Thomas Kreis (University of Geneva, Geneva, Switzerland). We are grateful to Jon Clayton and Belinda Bullard (European Molecular Biology Laboratory, Heidelberg, Germany) for teaching us how to dissect IFM for immunoblot analysis. We are also indebted to the members of Professor Walter J. Gehring's laboratory (Biozentrum) for the use of their equipment and for their advice on all work involving flies. Last but not least, we thank Dr. Bernhard Heymann, Dr. Richard Kammerer (both Biozentrum), and Prof. Henry F. Epstein (Baylor College of Medicine, Houston, TX) for fruitful discussions and constructive comments on the manuscript. This work was supported by the Swiss National Science Foundation, the Canton Basel-Stadt, and the M.E. Müller Foundation of Switzerland.

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