

# *Ifm(2)2* Is a Myosin Heavy Chain Allele That Disrupts Myofibrillar Assembly Only in the Indirect Flight Muscle of *Drosophila melanogaster*

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**Abstract.** Using a combination of molecular and genetic techniques we demonstrate that *Ifm(2)2* is an allele of the single-copy sarcomeric myosin heavy chain gene. Flies homozygous for this allele accumulate wild-type levels of mRNA and protein in tubular muscle of adults, but fail to accumulate detectable amounts of myosin heavy chain mRNA or protein in the indirect flight muscle. We propose that the mutation interferes with either transcription of the gene or splicing of the primary transcript in the indirect flight

muscle and not in other muscle tissues. Biochemical and electron microscopic analysis of flies homozygous for this mutation has revealed that thick filament assembly is abolished in the indirect flight muscle resulting in the instability of wild-type thick filament proteins. In contrast, thin filament and Z disc assembly are marginally affected. We discuss a working hypothesis for sarcomere assembly and define an experimental approach to test the predictions of this proposed pathway for sarcomere assembly.

A combined genetic and developmental approach has contributed most to our understanding of sarcomere assembly (Shafiq, 1963; Auber, 1969; Waterston et al., 1980; Zengel and Epstein, 1980). In *Drosophila melanogaster*, ultrastructural analysis of assembling indirect flight muscle (IFM)<sup>1</sup> myofibrils has revealed that filaments first appear at the periphery of fusing myoblasts. These filaments are loosely associated with Z bodies, the direct precursors of the Z band (Shafiq, 1963; Auber, 1969). As development proceeds, sarcomeres are found with as few as 30 thick filaments in hexagonal array with thin filaments. The fibers continue to grow in length and in diameter with the addition of new thick and thin filaments at the periphery of the myofibril. The center of the growing myofibril always shows the hexagonal array of thick and thin filaments. The M line and H zone structures become apparent later in the differentiation of the muscle (Shafiq, 1963).

Genetic analysis of flightless mutants has suggested that separate hierarchies exist for filament assembly (Mogami and Hotta, 1981). The *rsd* mutant, an actin null allele that fails to synthesize the IFM-specific actin isoform, has profound effects upon sarcomeric structure and the accumulation of a subset of myofibrillar proteins. Thin filaments fail to assemble, but interestingly, thick filaments and Z disc-related structures are present (Mahaffey et al., 1985). These results have established that the assembly of thick filaments is independent of thin filament assembly.

Mogami and Hotta (1981) have reported the isolation of a mutation, *Ifm(2)2*, mapping within 0.007 map units of the single copy sarcomeric myosin heavy chain (MHC) gene on the second chromosome at band 36B (Bernstein et al., 1983; Homyk and Emerson, 1988) that possesses dominant flightless behavior and is homozygous viable. This mutation affects the accumulation of a different subset of proteins than the *rsd* mutation. One protein that fails to accumulate and was hypothesized to be the focus of the mutation is protein spot 185 (Mogami and Hotta, 1981). We have cloned the gene that encodes protein spot 185 (Falkenthal et al., 1984) and have determined that this protein is the IFM-specific myosin alkali light chain (MLC-ALK) isoform (Falkenthal et al., 1985, 1987). Significantly, the gene that encodes this protein maps to the third, not to the second, chromosome. These results suggest that the *Ifm(2)2* mutation, like *rsd*, indirectly affects the synthesis or accumulation of an entire set of associated myofibrillar proteins.

In this communication we demonstrate that *Ifm(2)2* is an allele of the single-copy sarcomeric MHC gene. This mutation affects MHC gene expression in the IFM, but has no detectable effects in other muscle types. There is no detectable synthesis of MHC protein or accumulation of MHC mRNA in the IFM. This mutant allele has profound effects upon myofibrillar assembly; no thick filaments are present, whereas assembly of thin filaments and Z discs is minimally affected. Even though other thick filament proteins, such as the myosin light chains, are synthesized, they fail to accumulate. We discuss the implications of these results and propose a model to define the interrelationship of thick filament, thin filament, and Z disc assembly.

1. *Abbreviations used in this paper:* IFM, indirect flight muscle; MHC, myosin heavy chain; MLC-1 or -2, myosin light chain-1 or -2; MLC-ALK, myosin alkali light chain; TDT, tergotrochanteral muscle.

## Materials and Methods

### Growth and Maintenance of Fly Stocks

Fly stocks were grown at 23°C on agar-cornmeal-based media (Lewis, 1960). Pupae were synchronized by flotation 5 h after puparium formation (Mitchell and Mitchell, 1964) and aged at 23°C until they reached stage P12i (Bainbridge and Bownes, 1981). Canton-S flies were used as wild-type controls. The *Ifm(2)2* mutation was induced by ethyl methanesulfonate on a Canton-S genetic background (Mogami and Hotta, 1981), and was obtained from Dr. S. Bernstein (San Diego State University, San Diego, CA). *Mhc<sup>1</sup>/SM3.Cy* was obtained from Dr. C. P. Emerson, Jr. (University of Virginia, Charlottesville, VA) (Mogami et al., 1986).

### Preparation and Electrophoresis of Muscle Homogenates

IFM were dissected from isolated thoraces with fine forceps in TBI buffer (80 mM potassium chloride, 16 mM sodium chloride, 5 mM magnesium chloride, 15 mM potassium phosphate, pH 7.0, 1% [wt/vol] polyethylene glycol 6000 [Bonner et al., 1984]). Tubular muscle was prepared from tissue obtained by pulling the mesothoracic leg and the attached tergotrochanteral muscle (TDT) from the thorax with fine forceps. Dissected muscle bundles were suspended in IEF buffer (O'Farrell, 1975) or SDS sample buffer (Laemmli, 1970). All muscle samples were homogenized with a Pestle mixer (Kontes Co., Vineland, NJ).

Muscle proteins were displayed in one dimension according to their  $M_r$  (Laemmli, 1970). Two-dimensional gel analysis was performed by the method of O'Farrell (1975) using a pH range of 4–7. The gels were stained either with Coomassie Brilliant Blue R or with silver as described by Oakley et al. (1980).

### In Vivo Labeling of Indirect Flight Muscle Proteins

Thoraces of stage P12i pupae (Bainbridge and Bownes, 1981) were injected with 20  $\mu$ Ci of [<sup>35</sup>S]methionine and incubated at 23°C for 2 h. The labeled IFM was dissected and lysed in IEF buffer by homogenization. After electrophoresis, the labeled proteins were detected by fluorography.

### Isolation and Electrophoresis of RNA

Total cellular RNA was isolated from the IFM of 20 adults and from 40 heads by homogenizing dissected muscle bundles or heads in 200  $\mu$ l of 0.10 M sodium chloride, 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.5% sodium lauryl sulfate. The sample was deproteinized by phenol extraction, and the nucleic acid was collected by ethanol precipitation. The RNA samples were subjected to electrophoresis in denaturing 1% agarose gels containing 2.2 M formaldehyde as described previously (Rozek and Davidson, 1983). After electrophoresis, the RNA was transferred to nitrocellulose by capillary action (Thomas, 1980). Prehybridization and hybridization with 10% dextran sulfate were performed as described previously (Mullins et al., 1978). After washing the nonhybridized labeled DNA from the filter, the filter was air dried and then exposed to rapid x-ray film (Eastman Kodak Co., Rochester, NY) at –80°C.

DNA hybridization probes were nick-translated to a specific activity of 10<sup>8</sup> dpm/ $\mu$ g with [<sup>32</sup>P]dCTP. The cDNA clones, CD201 and MLC2–13.1, were used to detect MHC and myosin light chain-2 (MLC-2) mRNAs, respectively. MLC2–13.1 is a full length MLC-2 cDNA clone, whereas CD201 contains MHC sequences corresponding to the 3' exons 13–16. CD201 was kindly provided to us by Dr. Charles P. Emerson, Jr.

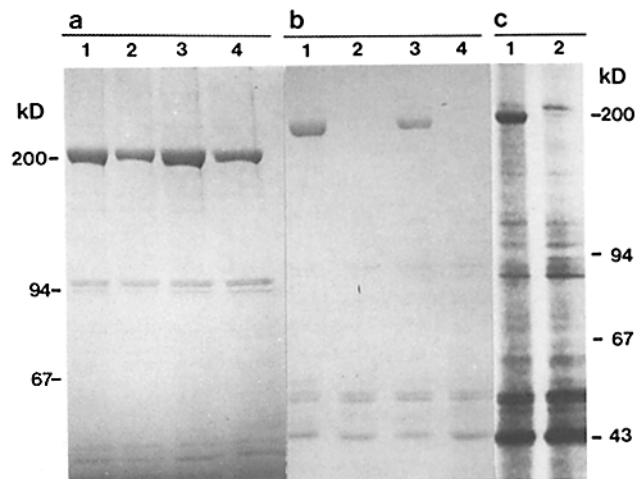
### Electron Microscopy

Thoraces were dissected from adult flies by cutting off the head and abdomen with a razor blade and pulling off the legs. The TDT muscle was removed with the mesothoracic legs. The thoraces were washed in 0.1 M cacodylate, pH 7.2, and then fixed in 3% glutaraldehyde in 0.1 M cacodylate, pH 7.2, for 2 h at room temperature. After fixing, the tissue was rinsed in 0.1 M cacodylate, pH 7.2, before the cuticle was teased away from the thorax. Next, the tissue was stained in 1% osmium tetroxide in 0.1 M cacodylate, pH 7.2, for 1 h at room temperature and then stained afterward in 2% aqueous uranyl acetate before dehydration. After dehydration, the tissue was washed in propylene oxide and then embedded in Epon. After polymerization, the Epon blocks were trimmed and the samples were sectioned using an ultramicrotome (MT-1; Sorvall Instruments, Newton, CT). Gold sections were collected and stained with 2% uranyl acetate and Reynold's lead citrate before viewing.

## Results

### *Ifm(2)2* Is a Mutation in the MHC Gene

*Ifm(2)2* maps within 0.007 map units of a *Drosophila* MHC null allele, *Mhc<sup>1</sup>*, which contains a deletion of intron 4 and exon 5 spanning nucleotides 4,155–4,255 3' from the transcription start site (Homyk and Emerson, 1988; O'Donnell and Bernstein, 1988). Because of the tight linkage of this mutation with the *Mhc<sup>1</sup>* allele (within 1,900 nucleotides assuming that 1 map unit approximates 275 kb of DNA [Kidd et al., 1983]), it is reasonable to suggest that *Ifm(2)2* is an *Mhc* allele. Therefore, we examined first the accumulation of MHC in the IFM of *Ifm(2)2* homozygotes. By visual inspection of the gel, MHC protein is undetectable in the *Ifm(2)2* lane (Fig. 1 b, lane 2). (This is not due to underloading the gel, because the amount of actin in the Canton-S lane is equal to that in the *Ifm(2)2* lane.) Moreover, a laser densitometric scan of the gel failed to reveal any accumulation of a 200-kD polypeptide, the  $M_r$  of MHC protein. No additional polypeptides to those detected in the Canton-S lane were present in the *Ifm(2)2* lane. This rules out the possibility that a stable, truncated form of the MHC protein accumulates in the IFM of *Ifm(2)2* homozygotes. As a control, we



**Figure 1.** SDS-polyacrylamide gel analysis of proteins that accumulate in tubular and IFM myofibers. Mesothoracic legs and the attached TDT were dissected from wild-type and mutant homozygotes, homogenized, and the solubilized proteins were displayed on a 7.5% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie Brilliant Blue. (a) TDT and mesothoracic leg muscles. Lane 1, wild-type; lane 2, *Mhc<sup>1</sup>/+*; lane 3, *Ifm(2)2*; lane 4, *Ifm(2)2/Mhc<sup>1</sup>*. Densitometric scans of the gels showed that the amount of MHC protein in the transheterozygote lanes (lanes 2 and 4) is 50% of wild-type. (b) IFM. Lane 1, wild-type; lane 2, *Ifm(2)2*; lane 3, *Mhc<sup>1</sup>/+*; lane 4, *Ifm(2)2/Mhc<sup>1</sup>*. (c). To detect the synthesis of MHC protein, in vivo <sup>35</sup>S-labeled proteins were displayed on a 7.5% SDS-polyacrylamide gel and were detected by fluorography. Lane 1, wild-type IFM (100,000 cpm); lane 2, *Ifm(2)2* IFM (100,000 cpm). In wild-type IFM, MHC protein ( $M_r = 200$  kD) is synthesized at a high rate. No detectable synthesis of a protein with a similar molecular mass to MHC is seen in *Ifm(2)2*. However, a band with a slightly slower mobility than MHC is detected. From laser densitometry, the intensity of this band is <2% that of the MHC band in the wild-type lane. If this is indeed MHC protein, it could represent a small amount of contamination of the IFM preparation with direct flight muscle or a very low rate of MHC synthesis in the mutant IFM.

examined the accumulation of MHC in tubular muscles of *Ifm(2)2* homozygotes. The MHC should accumulate in these muscles, because the tubular muscles are normal in their morphology and function. Significantly, the MHC protein accumulates to wild-type levels in the tubular muscles of *Ifm(2)2* homozygotes (Fig. 1 a).

Genetically, one can establish allelism of two mutations by a complementation test for function. However, because both mutations are dominant with respect to flightlessness and *Ifm(2)2* is homozygous viable, a complementation assay based upon function (viability or flight) could not be used. Rather, a molecular complementation assay was devised. *Ifm(2)2* heterozygotes accumulate MHC, whereas homozygotes fail to accumulate this protein; therefore, we can use the accumulation of MHC as an assay for complementation. If we assume that *Ifm(2)2* is not a *Mhc* allele, but perhaps an IFM-specific transregulatory factor of the MHC locus, then the transheterozygotes will be heterozygous at each genetic locus and will have the genotype *Mhc<sup>+</sup>Ifm(2)2<sup>+</sup>/Mhc<sup>+</sup>Ifm(2)2*. Therefore, adult flies should accumulate 25 and 50% of the wild-type concentration of MHC protein in the IFM and leg musculature, respectively. If, however, *Ifm(2)2* is a *Mhc* allele, the transheterozygotes will have the genotype *Ifm(2)2/Mhc<sup>+</sup>*. We expect that MHC should not accumulate in the IFM, but should accumulate in the TDT.

Transheterozygotes between *Mhc<sup>+</sup>* and *Ifm(2)2* were constructed by mating *Ifm(2)2* homozygous females with *Mhc<sup>+</sup>/SM3,Cy* males (Note, these males carry a balancer chromosome marked with the dominant mutation, curly wings). The transheterozygotes are easily identified because they have a *Cy<sup>+</sup>* phenotype (straight wings). The MHC content in the IFM of the transheterozygotes, as well as from *Ifm(2)2* homozygotes and *Mhc<sup>+</sup>/SM3,Cy* adults was assayed. As seen in Fig. 1 b, lane 3, the *Mhc<sup>+</sup>/SM3,Cy* heterozygote accumulates less MHC than Canton-S. Densitometry confirms this; the amount of MHC is 48% of wild-type, after normalizing to the amount of actin. The transheterozygotes fail to accumulate MHC proteins at detectable levels (<2% of wild-type) (Fig. 1 b, lane 4). As a control, the MHC content of the TDT of transheterozygotes was analyzed (Fig. 1 a). The accumulation of MHC protein in the TDT of *Ifm(2)2/Mhc<sup>+</sup>* transheterozygotes should be 50% of wild-type, because the *Ifm(2)2* mutation does not affect the accumulation of MHC protein in the TDT (Fig. 1 a), whereas the *Mhc<sup>+</sup>* null allele encodes no MHC protein in any tissue. Again, densitometry of the polyacrylamide gels confirms our prediction. On the basis of these results, we conclude that *Ifm(2)2* is an allele of the MHC gene.

#### Accumulation of MHC mRNA in *Ifm(2)2* Homozygotes

MHC protein fails to accumulate in the IFM of *Ifm(2)2* homozygotes. This could be due to two causes: (a) premature termination of translation resulting in the synthesis of an unstable truncated protein, or (b) no MHC mRNA is synthesized in *Ifm(2)2* IFM. Because we did not detect the synthesis of MHC protein or a novel protein species in the IFM of *Ifm(2)2* homozygotes (Fig. 1 c), it seemed probable that *Ifm(2)2* IFM lacks MHC mRNA. As shown in Fig. 2, MHC mRNA sequences are present in all tissues assayed except in the IFM of *Ifm(2)2* adults. Transcripts of 8.0 and 8.6 kb accumulate in the IFM of Canton-S, whereas transcripts of 8.0

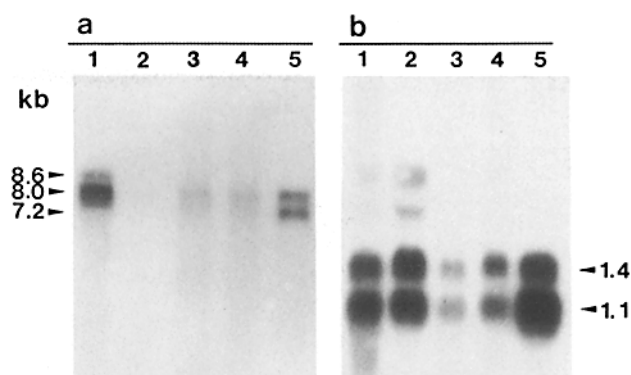


Figure 2. Accumulation of MHC and MLC-2 mRNA. RNA was isolated from IFM and heads. The RNA was subjected to electrophoresis in denaturing formaldehyde-agarose gels. After transfer to nitrocellulose, the presence of MHC and MLC-2 sequences was detected by hybridization with <sup>32</sup>P-labeled MHC (a) or MLC-2 (b) DNA. Hybridization was detected by exposure of the nitrocellulose filters to x-ray film. (a) MHC mRNA accumulation in various tissues. Lane 1, Canton-S IFM; lane 2, *Ifm(2)2* IFM; lane 3, Canton-S heads; lane 4, *Ifm(2)2* heads; lane 5, 72-h Canton-S larvae. (b) MLC-2 RNA accumulation in various tissues. Lane 1, Canton-S IFM; lane 2, *Ifm(2)2* IFM; lane 3, Canton-S heads; lane 4, *Ifm(2)2* heads; lane 5, 72-h Canton-S larvae.

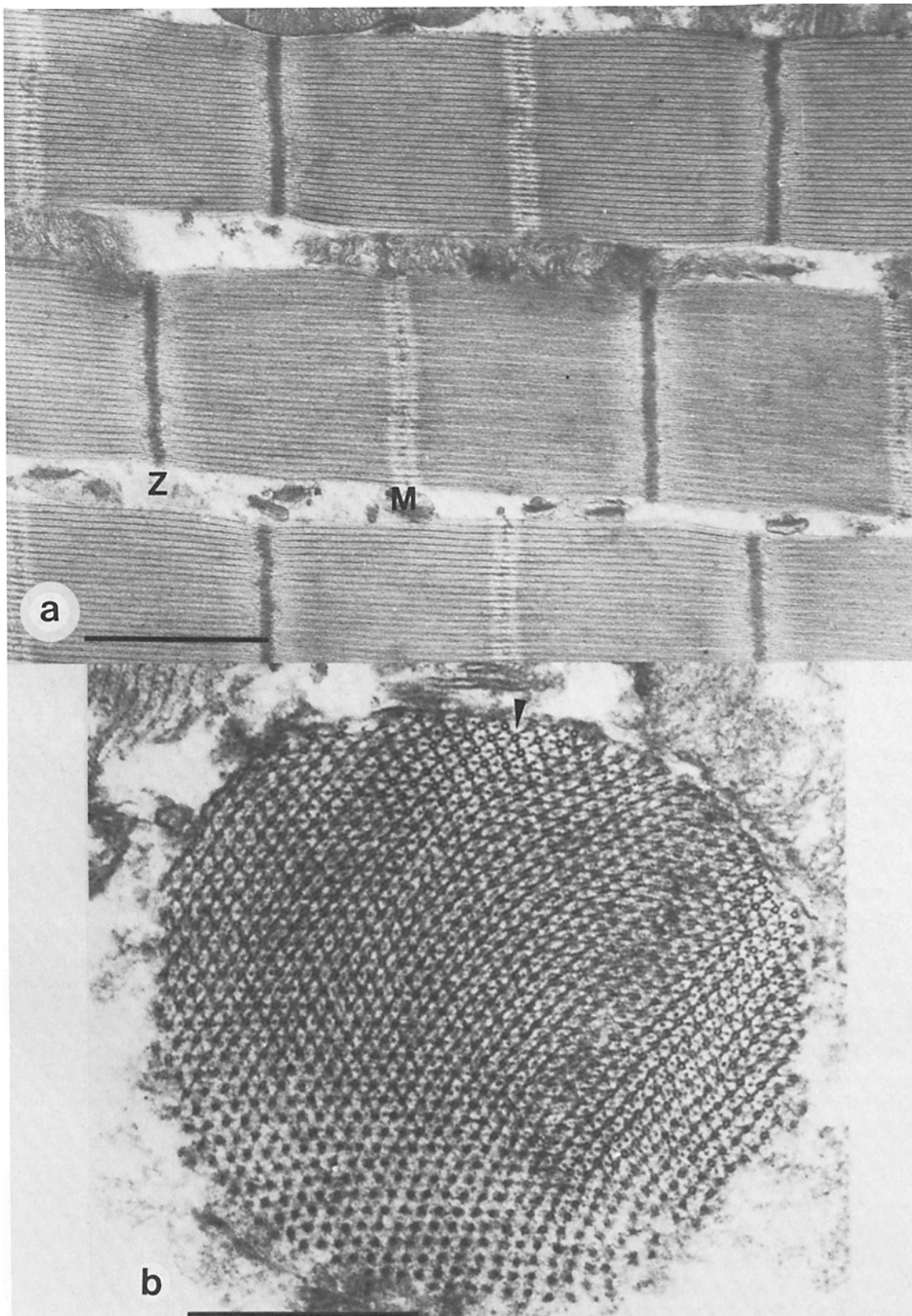
kb are detectable in the heads of both Canton-S and *Ifm(2)2*. As a control for the quality and quantity of RNA in each sample, the filter was stripped and rehybridized with <sup>32</sup>P-labeled MLC-2 sequences (Fig. 2 b). The concentration of MLC-2 mRNA is nearly equivalent between the Canton-S and *Ifm(2)2* IFM samples. Therefore, the failure to detect MHC mRNA sequences in *Ifm(2)2* is due to the absence of MHC mRNA in this tissue.

#### Myofibrillar Structure and Sarcomeric Organization of *Ifm(2)2*

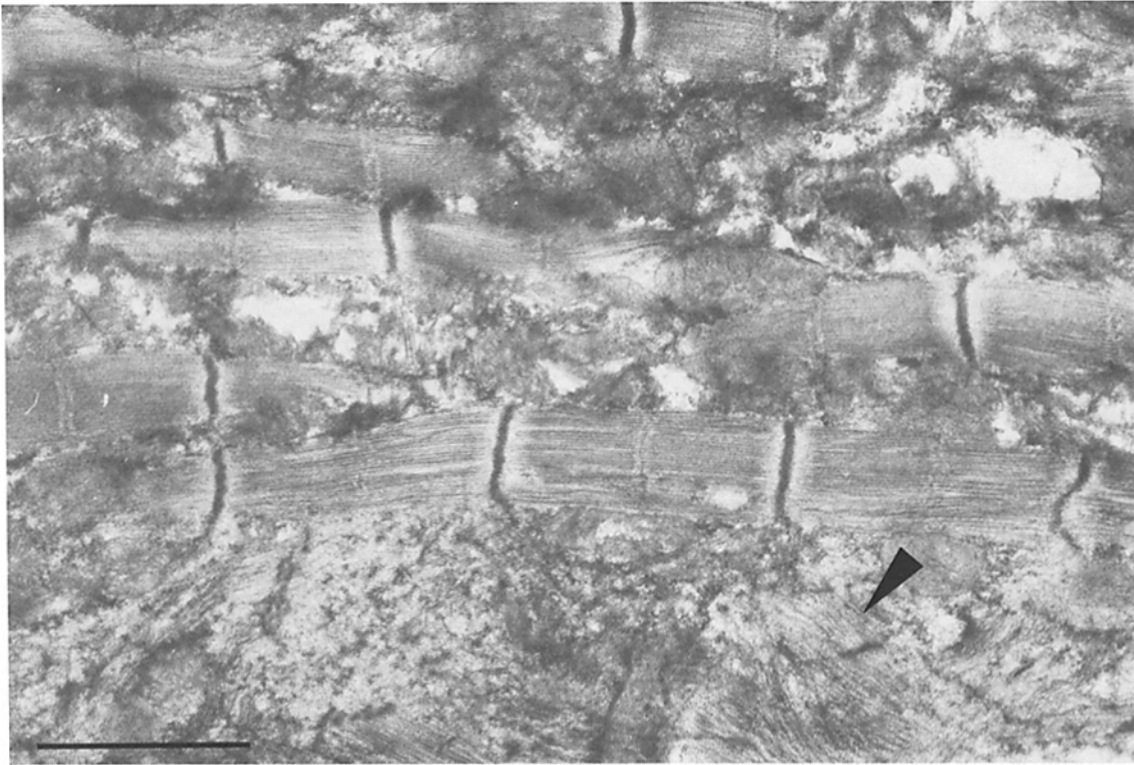
Examination of the IFM from *Ifm(2)2* homozygotes by phase-contrast microscopy revealed a complete lack of myofibrils (data not shown). To further understand this lack of sarcomeric structure, the IFM of wild-type and mutant flies were examined by electron microscopy. The myofibrils of wild-type IFM show a constant sarcomeric length and diameter of 2.7 and 1.1 μm, respectively (Fig. 3). The Z discs are the most electron-dense structure of the myofibril, appearing very straight and compact. In cross section, myofibrils show the regular hexagonal array of thin filaments surrounding each thick filament (Fig. 3 b).

By comparison to wild-type, the sarcomeric structure of *Ifm(2)2/+* heterozygotes is clearly disrupted (Fig. 4). In the most highly disorganized sarcomeres, the length of 3 μm is fairly constant; however, it is longer than wild-type length of 2.7 μm. In addition, the thick and thin filaments splay away from the longitudinal axis of the fibril. The Z discs are abnormal; they curve at the periphery of the fibril and are less electron dense than in the wild-type myofibril. The M line is present only in the middle section of the myofibril (Fig. 4). The sarcomeric organization is completely lost at the periphery showing a paucity of thick filaments and bundles of thin filaments (arrow, Fig. 4).

In stark contrast to the wild-type and *Ifm(2)2* heterozygotes, the IFM of *Ifm(2)2* homozygotes lack sarcomeric or-



*Figure 3.* Ultrastructure of wild-type IFM myofibrils. (a) Longitudinal section; (b) cross section. Note the regular hexagonal array of thick and thin filaments (arrowhead, b). Each sarcomere contains a well-defined Z disk (Z) and M line (M). Bars: (a) 1  $\mu\text{m}$ ; (b) 0.5  $\mu\text{m}$ .



**Figure 4.** Ultrastructure of IFM of *Ifm(2)2* heterozygotes. Longitudinal section of *Ifm(2)2* heterozygote. The arrowhead points to bundles of thin filaments found near the periphery of the muscle. Note that the sarcomeres are disorganized; in particular, the thick and thin filaments splay at the periphery of the fibrils, and the Z discs are curved. The M line is apparent only in the central region of the fibril. Bar, 2  $\mu$ m.

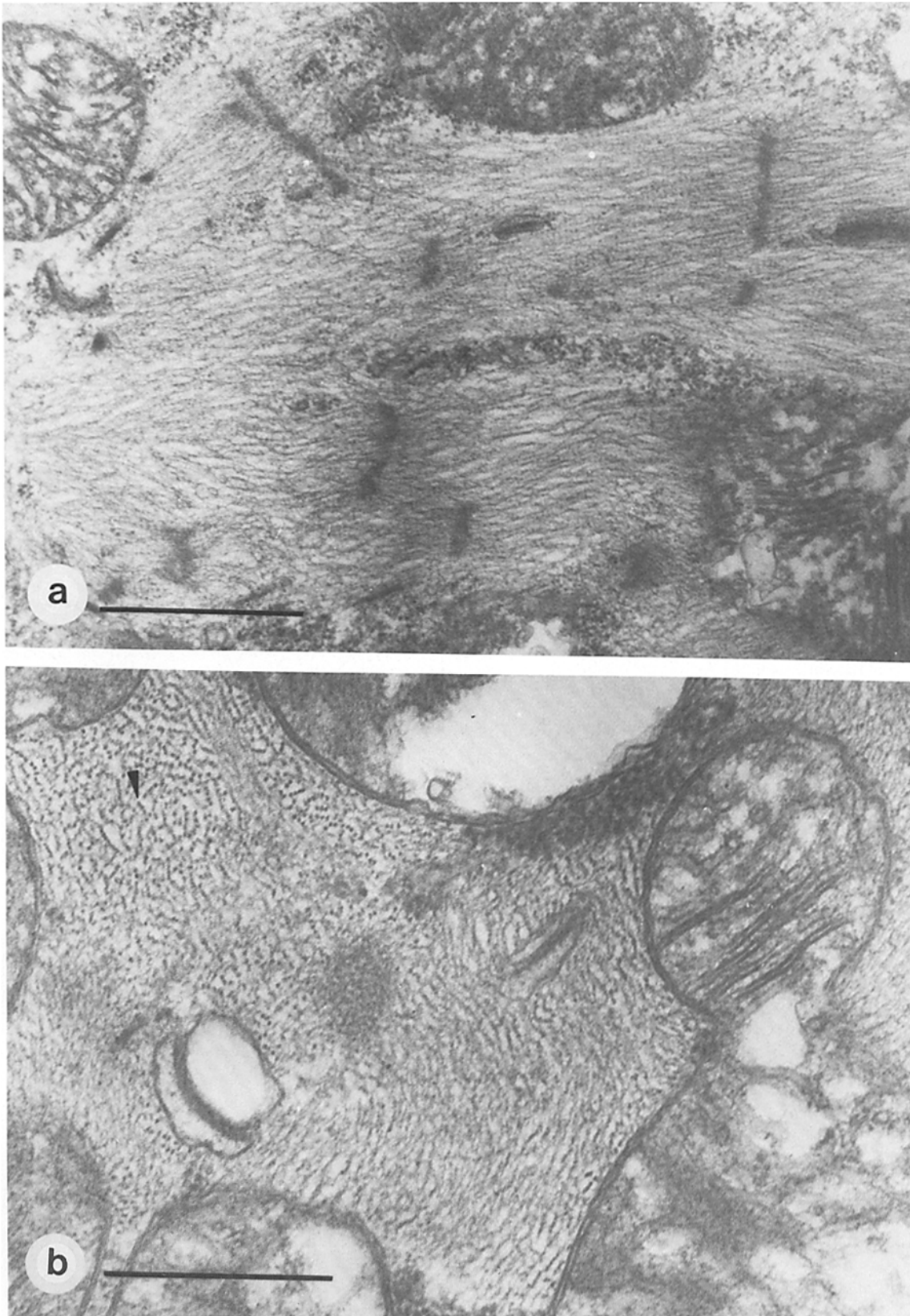
ganization (Fig. 5). Most notable is the complete absence of thick filaments throughout the muscle. Apparently normal thin filaments are surrounded by mitochondria. There is an increase in electron density along the length of the thin filaments with an approximate periodicity of 30–40 nm. In general, the majority of thin filaments maintain their normal arrangement along the longitudinal axis of the fiber and emanate from electron-dense material that appears to correspond to Z discs. However, the Z disc-like structures show a range of lengths and are no longer uniformly perpendicular to the longitudinal axis of the fiber. Therefore, the assembly of thin filaments and Z discs is affected in this mutant, but not to the extent of thick filament assembly, which is totally abolished.

#### **Accumulation of IFM Myofibrillar Proteins**

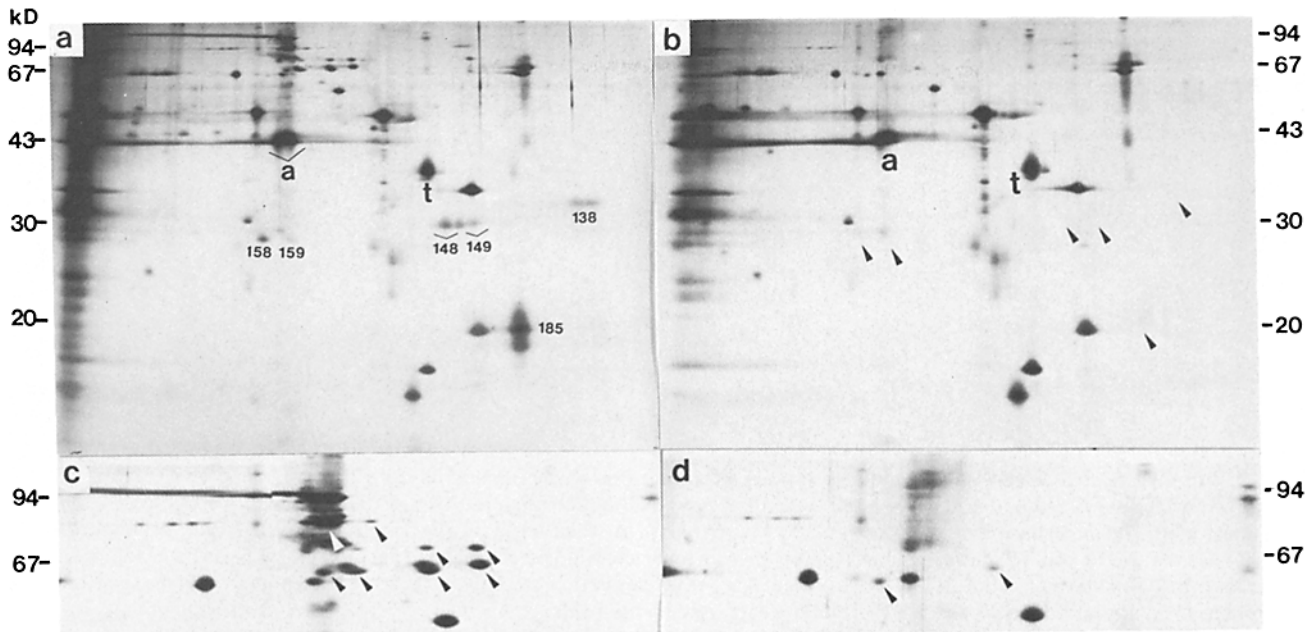
Previous studies of the proteins that accumulate in thoraces of *Ifm(2)2* homozygotes (Mogami and Hotta, 1981) indicated that IFM specific proteins 138 (myosin light chain-1 [MLC-1]) (Takano-Ohmura et al., 1983), 158, 159, and 185 (the IFM-specific MLC-ALK, [Falkenthal et al., 1987]) fail to accumulate. However, thoraces are composed of tubular muscle (the direct flight and TDT muscle) as well as the fibrillar IFM, and many myofibrillar proteins are common to both muscle types. We know that the function of the IFM is affected due to the lack of thick filaments. This gave us the opportunity to determine the molecular consequences of the absence of MHC upon the accumulation of not only the myosin light chains but other myofibrillar proteins.

A number of proteins present in the IFM of Canton-S fail to accumulate in the IFM of *Ifm(2)2* homozygotes (Fig. 6). In addition to the absence of the MLC-ALK protein (185), MLC-1 protein (138), proteins 158 and 159, there is a total absence of MLC-2 protein (148 and 149). Furthermore, >10 proteins with isoelectric points between that of actin (pI, 5.8) and tropomyosin (pI, 5.3) and molecular masses between 67 and 110 kD (Fig. 6, *c* and *d*) fail to accumulate. The accumulation of the major thin filament proteins actin and tropomyosin is equivalent in Canton-S and *Ifm(2)2* homozygotes. When two-dimensional gels of tubular muscles (mesothoracic leg and attached TDT) from Canton-S and *Ifm(2)2* homozygotes were compared, there were no quantitative or qualitative differences in the proteins accumulated (data not shown).

Is the failure of the proteins to accumulate in the IFM of *Ifm(2)2* homozygotes, as shown above, due to changes in protein synthesis or protein stability? We addressed this question by pulse labeling pupal *Ifm(2)2* homozygotes and Canton-S controls in vivo and visualizing the proteins synthesized in the IFM on two-dimensional polyacrylamide gels (Fig. 7). The pattern of proteins synthesized during the labeling period is indistinguishable between the mutant and control pupae. In particular, we note that the labeling of the MLC-2 protein relative to the labeling of actin is nearly equivalent in both wild-type and mutant myofibrils. Although we fail to detect the accumulation of the myosin light chains in the IFM of *Ifm(2)2* homozygotes on silver-stained



**Figure 5.** Ultrastructure of *Ifm(2)2* homozygotes. (a) Longitudinal section of *Ifm(2)2* homozygote. There is no sarcomeric organization. Note that thin filaments emanate from the electron-dense Z disk-like structures and that no thick filaments are apparent. (b) Cross section of *Ifm(2)2* homozygote. The arrowhead shows a cluster of thin filaments. Bars: (a) 1  $\mu\text{m}$ ; (b) 0.5  $\mu\text{m}$ .



**Figure 6.** Two-dimensional gel analysis of IFM proteins in wild-type and *Ifm(2)2*. Proteins from dissected IFM of 2-d adults were displayed on two-dimensional gels. The pH range for the first dimension is pH 4–7. The proteins were visualized by silver staining (Oakley et al., 1980). (a) IFM proteins of wild-type adults; (b) IFM proteins of *Ifm(2)2* homozygotes. The arrowheads in b denote the position of proteins present in wild-type myofibrils that are absent in the muscles of *Ifm(2)2* homozygotes. (c) An expanded view of the 55–110 kD molecular mass range of the gel shown in a. The arrowheads denote those proteins which are present in wild-type but absent in *Ifm(2)2* IFM. (d) An expanded view of the 55–110 kD molecular mass range of the gel shown in b. The arrowheads denote the position of two new protein spots not seen in the wild-type gel. a, Actin; t, tropomyosin.

gels, the rate of synthesis of these proteins appears to be similar to that of wild-type. From these findings we conclude that the accumulation of these proteins is absolutely dependent upon their assembly into thick filaments.

## Discussion

### The Primary Genetic Defect of the *Ifm(2)2* Mutation

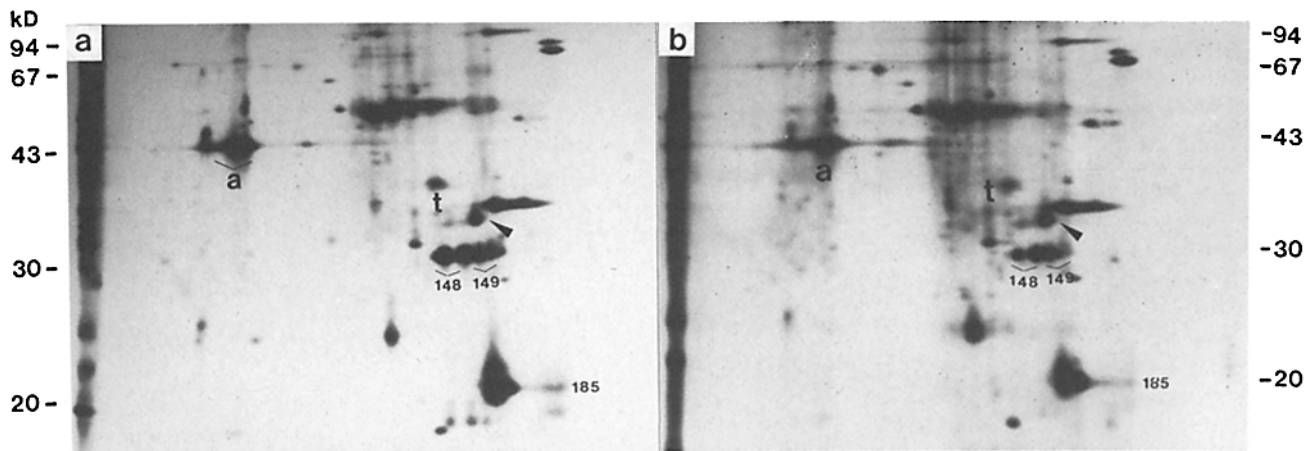
Our results, in addition to the recombination mapping which placed the *Ifm(2)2* mutation within 1.9 kb of the *Mhc*<sup>1</sup> mutation (Homyk and Emerson, 1988), indicate that *Ifm(2)2* is a tissue-specific null allele of the muscle-specific MHC gene which fails to synthesize MHC mRNA and protein only in the IFM. The other muscles are unaffected by this mutation. How can we explain the specificity of the *Ifm(2)2* mutation? The MHC gene undergoes alternative splicing at both the 5' and 3' ends of the gene generating different protein isoforms (Bernstein et al., 1986; Rozek and Davidson, 1986; Wassenberg et al., 1987; C. P. Emerson, Jr., personal communication). Therefore, the mutation may map to either the 3' or 5' end of the gene, interfering with the processing of the MHC primary transcript only in the IFM. In *C. elegans*, *unc 54* nonsense mutations result in significantly decreased levels of *unc 54* mRNA (Dibb et al., 1985). Therefore, it is formally possible that *Ifm(2)2* is a nonsense mutation that maps to an IFM-specific exon. An alternative proposal is that the *Ifm(2)2* mutation eliminates transcription of the MHC gene in the IFM, but not in other muscle tissues. Molecular characterization of upstream regulatory elements required for MHC expression in the IFM and of the tissue-specific splicing

of MHC transcripts in the IFM of wild-type and *Ifm(2)2* homozygotes will be required to establish the validity of each proposal.

### Pleiotropic Effects of *Ifm(2)2* on Thick Filament Assembly

In their original description of the *Ifm(2)2* mutation, Mogami and Hotta (1981) noted that a number of myofibrillar proteins fail to accumulate. Based on our observation that thick filaments fail to assemble, we predicted that the MHC and MLC-2 proteins would fail to accumulate as well. Upon examination, we were struck by the large number of proteins that were undetectable on silver-stained two-dimensional gels. Not only were the MHC and myosin light chain proteins missing, but >10 proteins between 67 and 110 kD fail to accumulate in the IFM. Not all of these proteins are IFM-specific (Mogami et al., 1982). We propose that some of these proteins are thick filament-associated; likely candidates are the thick filament-associated proteins myosin light chain kinase, phosphatase, and paramyosin. In addition, some of the missing proteins may be localized to the M line.

There are a number of possible causes for the failure of thick filament-associated proteins to accumulate. Either the mRNAs encoding these proteins are not synthesized in *Ifm(2)2* IFM, or the proteins are synthesized but then degraded in the absence of assembly. The *in vivo* labeling experiments and Northern gel analysis established that the MLC-2 mRNA and protein is synthesized; however, the MLC-2 protein is degraded rapidly in the absence of assembly.



**Figure 7.** Two-dimensional gel analysis of proteins synthesized in the IFM of developing pupae. [<sup>35</sup>S]Methionine was injected into the thoraces of developing pupae. After 2 h, the IFM were dissected, and the labeled proteins (250,000 cpm/gel) were displayed on two-dimensional gels. The labeled proteins were detected by fluorography. (a) Wild-type; (b) *Ifm(2)2*. a, Actin; t, tropomyosin. MLC-ALK (spot 185) and MLC-2 (spots 148 and 149), actin and tropomyosin represent a large percentage of the proteins synthesized in both wild-type and mutant IFM. We do not detect the synthesis of a protein that comigrates with MLC-1 spot 138; however, a protein with the same molecular mass but a slightly more basic pI is synthesized at a high rate (arrowheads).

*Drosophila*, mammalian, and bacterial cells all possess an energy-dependent intracellular protein degradation system (Arrigo et al., 1988; Falkenburg et al., 1988). In vitro as well as in vivo experiments suggest that the stable form of nonpolymerized tubulin is a dimer composed of an  $\alpha$ - and a  $\beta$ -tubulin subunit. Mutations that affect  $\beta$ -tubulin structure in Chinese hamster ovary cells result in assembly-defective  $\beta_1$ -tubulin subunits which are rapidly degraded with a half life of 1–2 h. These mutations also affect the accumulation of  $\alpha$ -tubulin suggesting that excess  $\alpha$ -tubulin is also degraded (Boggs and Fernando, 1987). Similar results have been observed in *Drosophila* for mutant  $\beta$ -2 tubulin subunits; the variant  $\beta$ -2 tubulin, as well as the wild-type  $\alpha$  subunit, is degraded (Kemphues et al., 1982). These examples suggest that proteolytic-sensitive sites are masked by either other proteins in the multimeric complex or conformational changes induced by assembly. However, in the absence of assembly these sites are not masked and the protein is degraded. This represents a simple means by which a cell can regulate the level of unassembled subunits without affecting transcription or translation.

#### **Sarcomere Assembly in the Absence of MHC Protein**

Previous developmental studies have not addressed the interdependency of thick filament, thin filament, and Z band assembly (Shafiq, 1963). It is remarkable that, in the absence of thick filaments, assembly of thin filaments and Z band structures occurs in the IFM of *Ifm(2)2*. Interestingly, the thin filaments align normally along the longitudinal axis of the IFM maintaining their attachments to Z band-like structures. These structures are reminiscent of the IZI structures obtained from myosin-extracted *Lethocerus* myofibrils (Goll et al., 1977). We noted a periodicity in electron density of 30–40 nm along the thin filaments. Negative staining of *Lethocerus* thin filaments also revealed projections showing a periodicity of 39 nm; this periodicity corresponds to a half turn of the thin filament axial repeat (Bullard, 1984). Because the troponin complex in *Lethocerus* is very large

(twice the molecular mass of vertebrate troponin), the projections certainly must be troponin (Bullard, 1984). Therefore, the “bumps” that we see along the *Drosophila* thin filaments most likely are troponin complexes reflecting the periodicity of tropomyosin along the thin filament. Interestingly, three-dimensional reconstruction of insect flight muscle in rigor revealed that cross-bridges occur with a periodicity identical to that of troponin. This analysis also revealed that thin filament structure is not uniform along the long axis of the myofibril showing an undertwist zone with maximal separation of the actin strands at the lead chevron (Taylor et al., 1984). The question remains as to whether the myosin heads contribute to the periodic change in the variable twist of the actin helix or whether this is innate to the structure of insect thin filaments. The absence of thick filaments in the IFM of *Ifm(2)2* should facilitate the isolation of sufficient quantities of native thin filaments for biochemical and structural analysis without having to resort to the harsh extraction procedures typically used for the isolation of thin filaments from *Lethocerus* asynchronous muscle.

The alignment and spacing of the Z band-like structures is altered in *Ifm(2)2* implying that thick filaments are not required to initiate the assembly of proteins into the Z band, while they are required for the completion of Z band assembly including the proper registry and alignment of the Z bands along the longitudinal axis of the myofiber. The actin null mutation, *rsd*, shows complementary effects upon filament assembly. In *rsd* homozygotes the IFM totally lacks thin filaments and Z bands; however, thick filaments as well as Z bodies assemble (Mahaffey et al., 1985) demonstrating that thin filaments are necessary for the differentiation of Z bodies to Z bands, but not for thick filament assembly. We do not know if thick filaments play a role in the stabilization of the Z body structure. Our working hypothesis is that there are three structures whose assembly is independent, but interrelated: the Z body, thick filaments, and thin filaments. Thin filament assembly influences the further structural modification of the Z body to the Z band (Shafiq, 1963; Ma-



haffey et al., 1985); whereas, thick filaments are necessary for the further elaboration of the H zone and the M line, and proper registry of the Z bands along the longitudinal axis of the fiber. We are currently testing this hypothesis by analyzing assembly during the development of the double mutant *Ifm(2)2; rsd*, which should lack both thick and thin filament systems. It is conceivable that Z bodies will assemble and accumulate in this genetic background.

Finally, because *Ifm(2)2* is a tissue-specific null allele of the MHC gene, flies carrying this mutation possess an excellent genetic background for transformation experiments with in vitro mutagenized MHC genes. Not only can the nucleotide sequences necessary for MHC transcription and RNA processing in the IFM be defined, but the amino acid sequences necessary for the stable assembly of the myosin light chains with the MHC can be determined. It is our hope that these experiments will further our understanding of sarcomere assembly and function.

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