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Transformation of *Drosophila melanogaster* with the Wild-type Myosin Heavy-chain Gene: Rescue of Mutant Phenotypes and Analysis of Defects Caused by Overexpression

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Abstract. We have transformed *Drosophila melanogaster* with a genomic construct containing the entire wild-type myosin heavy-chain gene, *Mhc*, together with ~9 kb of flanking DNA on each side. Three independent lines stably express myosin heavy-chain protein (MHC) at approximately wild-type levels. The MHC produced is functional since it rescues the mutant phenotypes of a number of different *Mhc* alleles: the amorphic allele Mhc^1, the indirect flight muscle and jump muscle-specific amorphic allele Mhc^{10}, and the hypomorphic allele Mhc^2. We show that the Mhc^2 mutation is due to the insertion of a transposable element in an intron of *Mhc*.

Since a reduction in MHC in the indirect flight muscles alters the myosin/actin protein ratio and results in myofibrillar defects, we determined the effects of an increase in the effective copy number of *Mhc*. The presence of four copies of *Mhc* results in overabundance of the protein and a flightless phenotype. Electron microscopy reveals concomitant defects in the indirect flight muscles, with excess thick filaments at the periphery of the myofibrils. Further increases in copy number are lethal. These results demonstrate the usefulness and potential of the transgenic system to study myosin function in *Drosophila*. They also show that overexpression of wild-type protein in muscle may disrupt the function of not only the indirect flight but also other muscles of the organism.

MYOSIN heavy-chain (MHC)[1] is one of the most abundant proteins in muscle. It has both a structural role in the thick filament and produces motile force while interacting with the thin filament (for review see Warrick and Spudich, 1987). Different isoforms of MHC are found in different muscle types within most organisms (for review see Emerson and Bernstein, 1987). The manner in which MHC amino acid sequence variation affects muscle structure and function in these different muscles is largely unclear, but this can best be addressed using organisms which can have their endogenous *Mhc* genes replaced by genes expressing different or altered MHC isoforms.

Foremost among the muscles amenable to genetic study are those of *Drosophila melanogaster*. There are a number of different muscle types in *Drosophila*, including the fibrillar indirect flight muscles, the tubular leg and direct flight muscles, and the supercontractile muscles of the body wall and gut. Each of these physiologically distinct muscles vary widely in their protein isoform usage (for review see Bernstein et al., 1993). Both viable and lethal mutations affecting muscle may be isolated, since mutations affecting only the indirect flight muscles (IFMs) or the tergal depressor of the trochanter (TDT, or "jump") muscle do not result in lethality. Furthermore, a number of muscle protein genes that are expressed in the IFMs show haploinsufficiency for flight, where a reduction in the amount of a particular protein affects muscle structure sufficiently to disrupt flight, but does not affect viability. This is true for the thick filament proteins MHC (Mogami and Hotta, 1981; Mogami et al., 1986) and myosin light-chain 2 (Warmke et al., 1988), and those in the thin filament such as actin (Mogami and Hotta, 1981; Beall et al., 1989) and tropomyosin (Mogami and Hotta, 1981; Karlik and Fyrberg, 1985). The sensitivity of IFM function to reductions in muscle protein gene dosages has been used as a criterion for identifying genes encoding myofibrillar proteins (Mogami and Hotta, 1981; Cripps et al., 1994). Beall and co-workers (1989) have shown that the phenotypes caused by haploinsufficiency for either *Mhc* or the *Act88F* actin gene likely result from nonstoichiometric levels of muscle proteins, since concomitant reductions in the levels of both actin and myosin restore a more normal myofibrillar structure and some degree of flight ability.

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1. *Abbreviations used in this paper*: IFM, indirect flight muscle; MHC, myosin heavy chain; TDT, tergal depressor of the trochanter.

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In *Drosophila melanogaster* a single copy gene, *Mhc*, gives rise to all of the muscle MHC (Bernstein et al., 1983; Rozek and Davidson, 1983), and different isoforms of the protein result from alternative splicing of the primary transcript (Bernstein et al., 1986; Rozek and Davidson, 1986; Wassenberg et al., 1987; George et al., 1989; Collier et al., 1990; Kronert et al., 1991). There is thus the potential for two classes of *Mhc* allele. Mutations in one class affect constitutive exons and therefore affect all muscles. The second class of mutations affect alternative exons and result in defects in a particular subset of muscles. Most of the mutations that have been characterized in the latter class disrupt IFM and occasionally TDT function, and are homozygous viable (Chun and Falkenthal, 1988; O'Donnell et al., 1989; Collier et al., 1990; Kronert et al., 1991, 1994).

D. melanogaster is therefore a useful system in which to study the functional significance of different MHC isoforms. Mutations may be generated in vitro in alternative exons which will affect only the muscles which are not required for viability. These genomic constructs may be transformed into flies and used to determine if the transgenic protein can functionally replace the endogenous isoform in a mutant background. The main problem with this approach is that *Mhc* is large, spanning 22 kb of genomic DNA, and manipulation and transformation of such large genes is difficult. In this paper we describe the transformation of flies with a genomic construct containing the whole *Mhc* gene plus ~9 kb of flanking DNA on either side. The transformed gene can rescue the mutant phenotypes associated with different classes of *Mhc* allele, including Mhc^2 which we show results from a transposable element insertion close to exon 7d. We have also studied the effects of increased *Mhc* dosage upon muscle, and conclude that overabundance of wild-type MHC can interfere with muscle assembly and function in several different muscle types. Transgenic studies of myofibril assembly must therefore be carried out with close attention paid to the levels of protein produced.

Materials and Methods

Isolation of Genomic Clones

The genomic cosmid library of J. Tamkun (Tamkun et al., 1992), cloned into the pCoSpeR vector (Pirrotta, 1988), was screened for clones which contain the entire MHC gene and flanking DNA using standard procedures (Sambrook et al., 1989). A single clone, designated pWMHC, satisfied these criteria.

A library of *SM1/*Mhc^2 *pr px sp* genomic DNA was constructed by cloning genomic DNA, partially digested with EcoRI, into the lambda vector EMBL4 (Stratagene Corp., La Jolla, CA) and packaging the DNA into phage heads using Gigapack extract (Stratagene Corp.). Four identical positive clones from 50,000 plaques of the amplified library were digested with EcoRI and identified as Mhc^2 by restriction mapping and Southern analyses based upon the published restriction map of Mhc^2 (Mogami et al., 1986). Restriction fragments were subcloned and characterized by sequencing.

P Element–mediated Germline Transformation

Transformation was performed essentially as described by Rubin and Spradling (1982). The *P* element helper plasmid was Δ2-3 (Robertson et al., 1988). The concentrations of cesium-purified DNA injected were 1 mg/ml of pWMHC and 100 μg/ml of Δ2-3. To reduce shearing of the large cosmid during injection, needles of slightly larger bore than routinely used were employed for a large proportion of the time (Haenlin et al., 1985). We injected white-eyed flies of the genotype w^{1118}, and potential transformants were identified in the G1 generation as orange-eyed individuals. Homozygous lines were made by standard genetic techniques using the stock *w; SM1/Sco; TM2/MKRS*, a gift from Dr. Greg Harris (San Diego State University, San Diego, CA).

Electrophoresis

One-dimensional SDS-PAGE was performed as described by Laemmli (1970). For large gels, samples of six fly upper thoraces were homogenized in 150 μl sample buffer, 12 μl was loaded into each well of the gel. For minigels, the dorsal longitudinal IFMs from three flies were dissected from the body as described by Peckham et al. (1990), homogenized in 60 μl of sample buffer, and 5 μl of each sample was loaded. Gels were scanned using an LKB Ultroscan XL laser densitometer.

Fly Stocks and Crosses

All gene and chromosome symbols are as described by Lindsley and Zimm (1992). Convention for naming transgenic lines is from Ashburner (1989). Flies were grown on Carpenter's medium (Carpenter, 1950) either in 100 mm × 25 mm glass vials or in half pint glass milk bottles at 25°C. All crosses for flight testing, SDS-PAGE, and electron microscopy consisted of two flies of each sex kept in vials at 25°C.

Potential lethal mutations other than Mhc^1 were removed from the Mhc^1 *pr px* chromosome as follows: females of the genotype *w/+;* Mhc^1 *pr px/ +++* were crossed to *w; SM1/+;* $P[w^+Mhc^+]wm3/MKRS$ males. Individual male progeny of the genotype *w; ???/SM1;* $P[w^+Mhc^+]wm3/+$ were first backcrossed to virgin female *w/+; SM1/*Mhc^1 *pr px; +/MKRS* or *TM2* to confirm the presence of the Mhc^1 and/or associated mutations. Once the first cross was established, the males were removed and crossed to *w; SM1/ Sco; TM2/MKRS* virgin females in order to make a stock either of *w; SM1/* Mhc^1 or of *w;* Mhc^1*;* $P[w^+Mhc^+]wm3$. Of 68 recombinant males tested, 17 died before the crosses were completed. One of the males gave rise to a line in which the second chromosome was homozygous viable in the presence of either one or two copies of the $P[w^+Mhc^+]wm3$ chromosome, but was otherwise homozygous lethal. The presence of an Mhc^1 allele in the new *w; SM1/*Mhc^1 stock was further confirmed by PCR analysis of genomic DNA. Primers flanking the region deleted in the Mhc^1 mutation gave rise to two PCR products, one from the transformed wild-type gene and one from the Mhc^1 mutant gene that differed in size from one another by ~100 bp (data not shown). This difference is the extent of the Mhc^1 deletion (Mogami et al., 1986; O'Donnell and Bernstein, 1988).

The $P[w^+Mhc^+]wm1$ and $P[w^+Mhc^+]wm3$ transgenes were crossed into an Mhc^{10} background using standard genetic techniques. The $P[w^+Mhc^+]$ *wm2* transgene, which resides on the same chromosome (chromosome II) as the endogenous *Mhc* gene, was recombined onto an Mhc^{10} chromosome by crossing *w;* Mhc^{10}*+/+*$P[w^+Mhc^+]wm2$ virgin females to *w; SM1/*Mhc^{10}*+* males. Individual red-eyed progeny were flight-tested and the flightless virgin females retained. These were of the genotype *w;* $Mhc^{10}P[w^+Mhc^+]$ *wm2/*Mhc^{10}*+*, and were crossed to *w; SM1/*Mhc^{10}*+* males to obtain *w; SM1/*Mhc^{10} $P[w^+Mhc^+]wm2$ males and females to make an homozygous stock. Note that we do not know the cytological location of the $P[w^+Mhc^+]$ *wm2* transgene and have arbitrarily listed it after *Mhc*.

Flight and Jump Testing

Flight testing was performed as described by Drummond et al. (1991), upon 1–2-d-old flies.

Jump testing was performed by removing the wings of 0–1-d-old flies and allowing them to recover overnight. The flies were then placed in the upturned lid of a plastic Petri dish raised 100 mm above paper coated with mineral oil, and encouraged to jump from the edge of the dish using a paintbrush. The landing point on the paper was marked, and the average horizontal distance from the edge of the Petri dish determined for each genotype.

Electron Microscopy

Female flies aged 1–2 d were prepared for electron microscopy using the procedures described by O'Donnell and Bernstein (1988).

Results

Isolation of a Genomic Mhc Clone

To obtain clones which contain the entire MHC gene as

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well as all sequences necessary for proper expression, we screened a genomic library made in a *P* element cosmid vector (Tamkun et al., 1992). Using fragments of DNA from both ends of *Mhc* to probe replica filters (plasmids pMHC1 and p9C3A; see Fig. 1 *a*), 41 clones containing some portion of *Mhc* were isolated. Six of the 41 clones hybridized to both probes, and were extensively restriction enzyme mapped and characterized. Only one clone, pWMHC, contained the entire gene and ~9 kb of 5′ and 3′ flanking sequences. Fig. 1 *a* shows an EcoRI restriction map of the whole clone, the transcribed region of *Mhc* within this, and DNA probes used to confirm that the gene is contiguous. In total, pWMHC is ~50-kb long.

Transformation of pWMHC

P element mediated germline transformation of DNA becomes increasingly difficult as the size of the transforming plasmid increases. Only a few reports of transformation with plasmids of approximately 50 kb exist (Haenlin et al., 1985; Ramos et al., 1989). We injected 6,043 embryos, of which 1,321 hatched, and 421 fertile adults eclosed. These gave rise to three independent transformed lines, designated *P[w⁺ Mhc⁺]wm1*, *wm2*, and *wm3*. Standard crosses revealed that *wm1* is on the X chromosome, *wm2* is on the second, and *wm3* is on the third chromosome.

To determine if each line arose from single independent insertional events, genomic DNA from the w^{1118} parental strain and from each of the three lines was digested with EcoRI, separated by agarose gel electrophoresis, and blotted onto nylon membrane. Hybridization with radioactively labeled probes made from the plasmids pL170-3 or pL170-1 (see Fig. 1 *a*) revealed a single band of hybridization in w^{1118}, corresponding to the region of the endogenous *Mhc* gene (Fig. 1 *b*). In the case of each transformed line, two bands of hybridization were observed: one the same size as that in the w^{1118} lane corresponding to the endogenous gene and one additional band, different in each lane, corresponding to the transformed genes inserted at different genomic locations (Fig. 1 *b*). Since there is only one additional band in each line, this indicates that each arose from a single insertional event. The use of probes from either end of the transformed gene confirms that none of the lines consist of two *P* elements inserted adjacent to one another.

Molecular Defect in Mhc²

We attempted to rescue the dominant flightless phenotypes of three different *Mhc* alleles in order to confirm that the transformed lines were expressing functional protein. We chose these three different alleles so that we could observe some aspects of rescue even if the transformed genes were not expressing at high levels or in all tissues. Two of these mutants have been characterized previously: *Mhc¹* is a null allele in which all classes of functional *Mhc* transcript are abolished (O'Donnell and Bernstein, 1988); and *Mhc¹⁰* is a null allele that lacks *Mhc* transcripts in the IFMs and the TDT muscles only (Collier et al., 1990). The third allele, *Mhc²*, is known to be hypomorphic since homozygous mutants do not die as embryos but survive to first instar larvae, indicating that some functional protein is produced from the mutant gene (Mogami et al., 1986). Also, whereas *Mhc¹*/+ heterozygotes display 64% of the wild-type thoracic MHC content, *Mhc²*/+ has 77%, showing that protein is also produced in adults (Mogami et al., 1986). We determined the

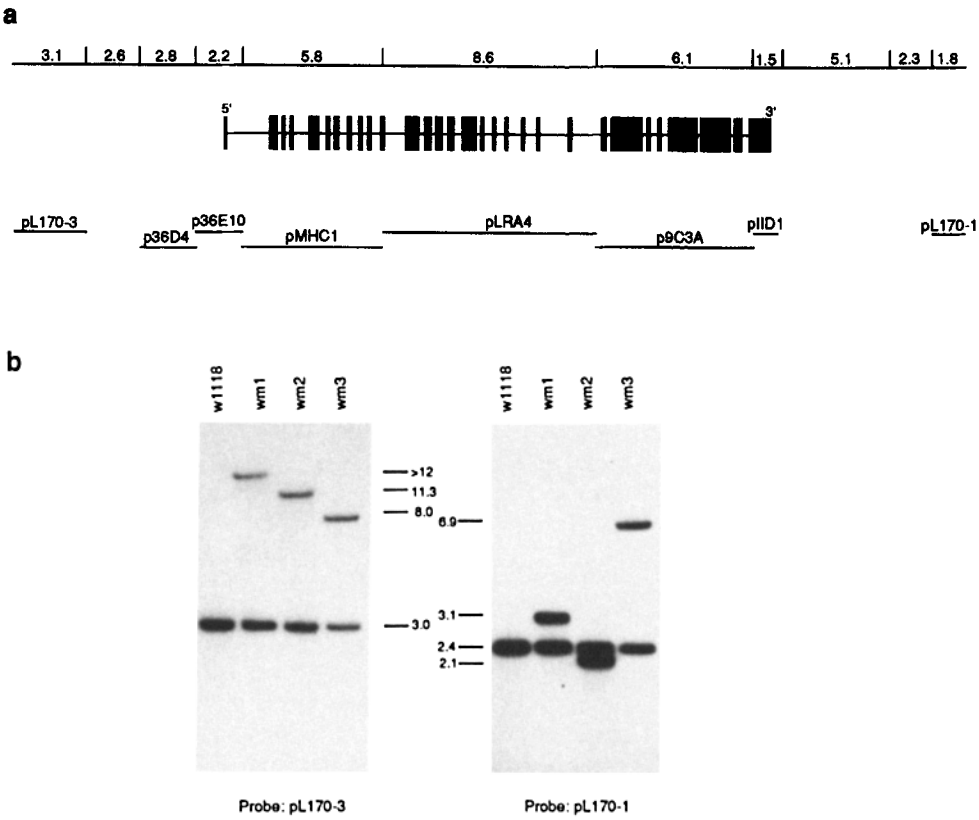

Figure 1. Structure of the cosmid clone pWMHC and Southern analysis of transformed lines. (*a*) The top line indicates the extent of pWMHC showing EcoRI restriction sites by vertical lines, and the sizes of each fragment in kb; below that, the exon–intron structure of *Mhc* within the cloned region is detailed; the bottom lines show the various plasmid clones used in this study. (*b*) Southern blot analysis of the three transformed lines using pL170-3 (*left*) and pL170-1 (*right*). There is only a single band of hybridization in the w^{1118} lanes corresponding to the endogenous *Mhc* gene, and two bands of hybridization in the other lanes corresponding to the endogenous plus inserted *Mhc* genes. Approximate band sizes in kb are indicated. wm1, wm2, and wm3 refer to the three transformed lines.

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a: Insertion site of *springer*

```
                                                    Exon 7d        ->
Mhc+                    TTTTCCAACTCTGTGTATGTAG AGATGTGCTTCCTCTC
                        :::::::::: ::::::
Mhc2                    TTTTCCAACTATGTGTAAGTTCAATAAGGATGCA
                                   :::: : ::::   :
Ifm(3)3 springer                   AGTTAACTAAGTTAACC
```

b: Sequence comparison

```
DROTN891   D. melanogaster springer copia-like 8.8 kb transposon
  86.8% identity in 266 nt overlap

              10         20         30         40         50
Mhc2    GAGCCTCTTCTCCTAGATT-GGAGATATGGTG---GAGCGTCTCT-CGTTGTTGACTGCC
        ::::::::::::::::: ::::::::::::   :: ::::::: ::::::::::::::
DROTN891 AGACCTCTTCTCCTAGATTGGGAGATATGGTGGGAGAACGTCTCTCCGTTGTTGACTGCC
             140        150        160        170        180        190

           60         70         80         90        100        110
Mhc2    CTTAAGGCTAGCCKACCGATTTCAATGATAACA-GCAG-TAGCTGGAGTT-GATTGAAKK
        ::::::::::::: ::: : :::::::::::::: :::: :::::::::::: :::::::::
DROTN891 CTTAAGGCTAGCCAACC-AATTCAATGATAACAGGCAGTTAGCTGGAGTTAGATTGAAGG
             200        210        220        230        240        250

                120        130        140        150        160
Mhc2    ---GATGCGCTC-TTTATT-GAATACAAAATCAAACTGACTATAAGCTAACAKKKKKAAA
           ::::::::: :::::: :::::: :::::::::::::::::::::::  :....  X:
DROTN891 CGCGATGCGCTCTTTTATTGGAATAC-AAATCAAACTGACTATAAGCTACAAGGG---AA
              260        270        280        290        300

        170        180        190        200        210        220
Mhc2    AACATCATAGCGGCCTCTGCCAATGCGCAGAGCTTCTGCCAACTATGCAGTGAGCTTCCG
        :::::::::::::::::::::::::::::::::::::::  ::::::X ::::::::::
DROTN891 AACATCATAGCGGCCTCTGCCAATGCGCAGAGCTTCTGCCGGCTATGCA-TGAGCTTCCG
        310        320        330        340        350        360

        230        240        250
Mhc2    GCCACAACTGCTTGGTCAGCAGTTTGACC
        ::  ::: :::::::::::::: :::::::
DROTN891 GC--CAAATGCTTGGTCAGCAATTTGACC
           370        380        390
```

Figure 2. The mutation in *Mhc²* is a *springer* element insertion upstream of exon 7d. (*a*) Sequence of *Mhc²* at the point of divergence from wild-type sequence; (*b*) Sequence comparison between the *springer* element in *Mhc²* (just upstream of an internal EcoRI site) and that in the *Ifm(3)3* mutation of *Tml* [DROTN891] (Karlik and Fyrberg, 1985).

molecular lesion in this allele in order to understand the possible basis of rescue by the transformed copy of *Mhc*.

Mhc² arises from a 10-kb insertion within *Mhc* (Mogami et al., 1986), resulting in an altered restriction map which was used to identify cloned fragments of the mutant gene. Sequencing showed that the mutant DNA diverged from the wild-type sequence 5-bp upstream of alternative exon 7d (Fig. 2 *a*). Sequencing a portion of the inserted element revealed homology to the *springer* family of copia-like transposable elements (Fig. 2 *b*). *springer*-induced mutations in *Drosophila* have been previously described in the *Tml* tropomyosin gene (Karlik and Fyrberg, 1985), the *Notch* gene (Kidd and Young, 1986), and the *forked* gene (Ishimaru and Saigo, 1993).

Mhc²/+ adults and larvae produce an abnormally small *Mhc* mRNA (Mogami et al., 1986). These authors suggested that this results from premature transcription termination within the *springer* element. The low level of MHC protein produced from *Mhc²* and the observed motility of homozygous *Mhc²* larvae indicate that transcription can occasionally proceed through *springer*, and that the intron including the element can be spliced out. Our proof that the insertion is in an intron gives further weight to this argument.

Since *springer* is inserted so close to exon 7d we determined if it affects the splicing of that particular exon. Exon 7d is normally included in transcripts in the IFMs (Hastings and Emerson, 1991); therefore we crossed *SM1/Mhc² pr px sp* to *Mhc¹⁰*, a viable *Mhc* allele in which no MHC is expressed in the IFM and the TDT muscles only (Collier et al., 1990). We collected *Mhc¹⁰/Mhc² pr px sp* adults to test for MHC accumulation in IFMs. For comparison, we also performed the same experiment using the *Mhc³* and *Mhc⁴* lethal alleles. The IFMs of these adults were dissected from the body with no contaminating muscles, and constituent proteins separated by one-dimensional SDS-PAGE. Since *Mhc¹⁰* is an IFM-specific amorphic allele, any MHC observed in the *Mhc¹⁰/Mhc²* samples must be from *Mhc²*. Comparison of the IFM proteins (Fig. 3, lanes *1–5*) in *Mhc¹⁰/Mhc²* (lane *3*) with *Mhc¹⁰/SM1* (lane *1*) and with *Mhc¹⁰* homozygotes (lane *2*) revealed that there is no detectable MHC in the *Mhc¹⁰/Mhc²* muscles. Thus, the presence of the *springer* element prevents the accumulation of normal exon 7d–containing transcripts. However, functional isoforms of MHC are produced using exons 7a, 7b, or 7c since homozygous *Mhc²* larvae are capable of movement and most hatch from the eggshell. Our results also show that low levels of MHC are produced in the IFM from *Mhc³* since there is a small amount of MHC visible in *Mhc³/Mhc¹⁰* (lane *4*), but that no detectable MHC is produced in the IFMs from *Mhc⁴* (lane *5*). We confirmed our conclusions regarding MHC accumulation in the IFMs of each mutant by western blot analysis of the same protein samples using an anti-MHC polyclonal antibody (Kiehart and Feghali, 1986) kindly provided by D. Kiehart (Duke University, Durham, NC) (data not shown). These data are consistent with those of Mogami et al. (1986) who showed that whole thoraces from *Mhc³*/+ adults have more MHC than whole thoraces from *Mhc²*/+ or *Mhc⁴*/+ flies.

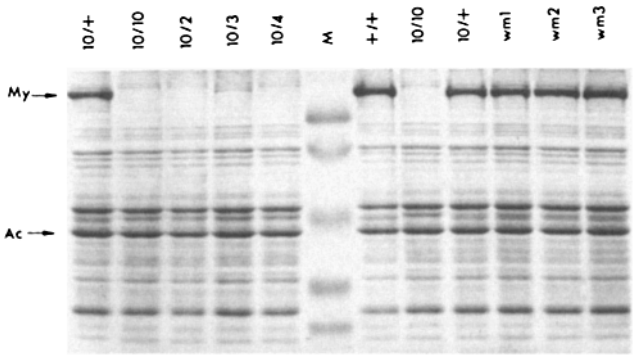

Figure 3. Accumulation of MHC expressed by *Mhc* mutants and the transformed genes in an *Mhc¹⁰* background. Lanes are as follows: *10/+*, *Mhc¹⁰*/+; *10/10*, *Mhc¹⁰* homozygotes; *10/2*, *Mhc¹⁰/Mhc²*; *10/3*, *Mhc¹⁰/Mhc³*; *10/4*, *Mhc¹⁰/Mhc⁴*; *M*, relative molecular mass markers (107, 76, 52, 37, 27 kD); +/+, wild-type; *10/10*, *Mhc¹⁰* homozygotes; *10/+*, *Mhc¹⁰*/+; *wm1*, *wP[w⁺Mhc⁺]wm1/+; Mhc¹⁰*; *wm2*, *Mhc¹⁰P[w⁺Mhc⁺]wm2/Mhc¹⁰*; *wm3*, *Mhc¹⁰*; *P[w⁺Mhc⁺] wm3/+*. *My*, myosin; *Ac*, actin.

Rescue of Dominant Flightless Phenotypes of Mhc Mutants

To determine if the transformed genes express functional protein, we crossed *SM1/Mhc¹* and *SM1/Mhc²* to *w¹¹¹⁸* and to the transformed lines to see if the dominant flightless phenotypes could be rescued. *Mhc¹*/+ and *Mhc²*/+ heterozygotes are flightless (Mogami et al., 1986; Table I). However, in the presence of an additional transgenic copy of *Mhc*

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Table I. Flight Abilities of Wild-type, Mutant, and Rescued Flies

Genotype	Number tested	Flight ability (percentage)			
		Up	Horizontal	Down	Not at all
Wild-type strains					
Canton-S	142	86	13	1	0
w^{1118}	133	85	2	8	5
Mhc^1/+ mutant and rescued					
Mhc^1/+	120	0	0	14	86
wP[w⁺Mhc⁺]wm1/+ or Y; Mhc^1/+	102	97	2	1	0
Mhc^1 *+/+P[w⁺Mhc⁺]wm2*	106	95	2	2	1
Mhc^1 */+; P[w⁺Mhc⁺]wm3/+*	107	94	6	1	0
Mhc^2/+ mutant and rescued					
Mhc^2/+	127	0	0	12	88
wP[w⁺Mhc⁺]wm1/+ or Y; Mhc^2/+	111	97	2	1	0
Mhc^2 *+/+P[w⁺Mhc⁺]wm2*	106	96	3	1	0
Mhc^2 */+; P[w⁺Mhc⁺]wm3/+*	111	94	5	1	0
Mhc^{10} mutant and rescued					
Mhc^{10}	108	0	0	4	96
wP[w⁺Mhc⁺]wm1; Mhc^{10}	66	50	21	18	11
w; Mhc^{10}*P[w⁺Mhc⁺]wm2*	143	22	9	49	20
w; Mhc^{10}*; P[w⁺Mhc⁺]wm3*	110	38	17	36	8
Mhc^1 homozygotes rescued					
w; Mhc^1*; P[w⁺Mhc⁺]wm3*	91	80	12	7	1

flight ability is fully rescued (Table I). Mhc^{10}/+ mutant phenotypes are also rescued by the presence of one of the transgenes (data not shown). We performed electron microscopy upon the IFMs of mutant and rescued flies to determine if abnormal myofibril structure is also rescued. Mhc^1/+ has disruptions in the structure of the IFM myofibrils (O'Donnell and Bernstein, 1988; Fig. 4 *a*) and Mhc^2/+ myofibrils are similar (Fig. 4 *c*), as would be expected since we have shown that no MHC is produced in the IFMs from Mhc^2. In transverse sections of the mutant muscles, cracks are visible throughout the myofibrils where no thick filaments are present and skeins of thin filaments can be seen. These cracks are also visible in longitudinal section, and may run the length of the sarcomere. When Mhc^1/+ or Mhc^2/+ also contain one copy of any one of the transformed genes, flies have normal IFM ultrastructure (Fig. 4 *b* and *d*). Clearly, sufficient protein is being produced from these transgenes to rescue structural and functional muscle defects caused by haploinsufficiency for *Mhc*.

Rescue of Recessive Phenotypes of Mhc^1 and Mhc^{10}

In order to ascertain the level of MHC expressed from the transformed genes, we crossed each of them into an Mhc^{10} background, such that all the MHC in the IFMs and the TDT is produced from the transformed genes (see Materials and Methods for details of crosses). One-dimensional SDS-PAGE of the IFM proteins of these lines (Fig. 3, lanes *7–12*) revealed that each gene produces approximately normal levels of MHC, since the level of MHC in Mhc^{10}/+ (lane *9*) is similar to that for Mhc^{10} homozygotes with one copy of any of the transformed genes (lanes *10–12*).

Although flies homozygous for both Mhc^{10} and for any of the transformed genes fly better than Mhc^{10} homozygotes, they do not fly as well as wild type (Table I). There are three possible reasons for this: the first is that insufficient levels of MHC are produced from the transgenes to fully rescue muscle function in Mhc^{10} homozygotes. This is unlikely, since SDS-PAGE (Fig. 3) reveals high levels of expression from the transgenes, and since the IFM ultrastructure of these flies is normal (Fig. 4 *f*) compared to that of Mhc^{10} homozygotes (Fig. 4 *e*; Collier et al., 1990). The second possibility is that the transgenes are not producing fully functional protein. This also seems unlikely given that the IFMs, which are most sensitive to changes in muscle protein gene dosage, are structurally normal, as are the IFMs of Mhc^1/+ and Mhc^2/+ that are rescued with the transgene. The final possibility is that although the IFMs are functionally normal, MHC is being overexpressed in other thoracic muscles important for flight such as the direct flight muscles. This might be expected since the Mhc^{10} mutation does not affect these muscles. Overexpression of MHC in the direct flight muscles may affect their function sufficiently to disrupt flight.

We also studied the jumping ability of Mhc^{10} homozygotes, and those rescued with the transgenes (Table II). Whereas Mhc^{10} homozygotes are unable to jump since no MHC is present in the TDT (Collier et al., 1990; Table II), *wP[w⁺Mhc⁺]wm1;* Mhc^{10} and *w;* Mhc^{10}*P[w⁺Mhc⁺]wm2* homozygotes both jump strongly. There is a marked difference in the jumping abilities of Canton-S and w^{1118} adults, indicating that this is a very variable behavior, but the rescued lines both jump as well as w^{1118}. Clearly muscle function is rescued in the TDT as well as in the IFM by the transgenes.

To test if sufficient levels of functional MHC are produced from a transformed copy of *Mhc* to rescue recessive lethality, *SM1/*Mhc^1 *pr px* and *SM1/*Mhc^2 *pr px sp* were crossed with w^{1118}*; P[w⁺Mhc⁺]wm3*-containing lines in an attempt to produce *w;* $Mhc^{1\ or\ 2}$*; P[w⁺Mhc⁺]wm3* flies. We chose the *wm3* line since it is on the third chromosome and is more easily manipulated with second chromosome *Mhc* mutations. Also, this obviates potential problems associated with dos-

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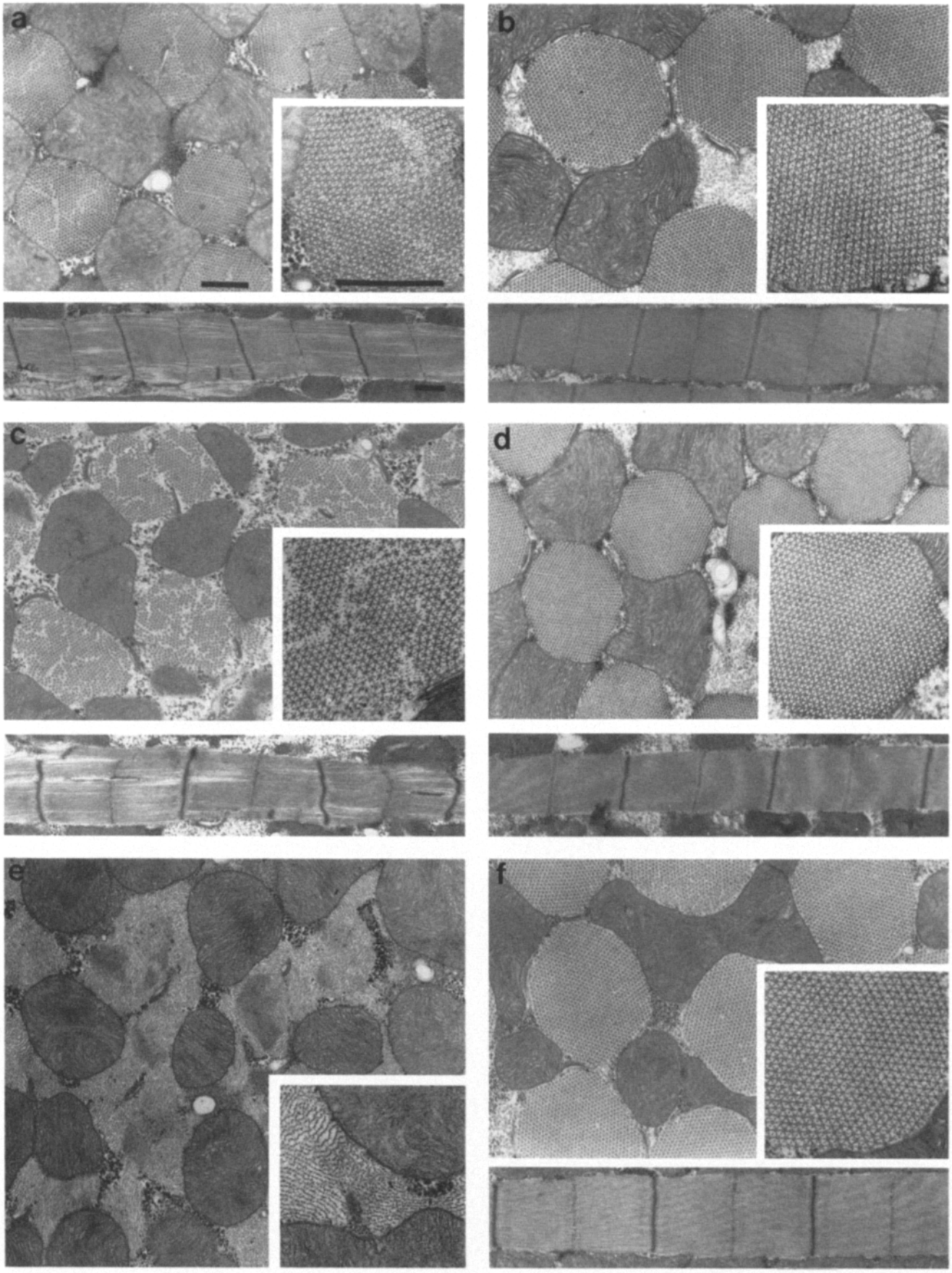

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Table II. Jumping Abilities of Mutant and Rescued Flies

Genotype	Sex	Average distance
		*mm**
Canton-S	Female	47 ± 6
Canton-S	Male	45 ± 5
w^{1118}	Female	28 ± 4
w^{1118}	Male	19 ± 5
Mhc^{10}	Female	5 ± 2
Mhc^{10}	Male	4 ± 1
$wP[w^+Mhc^+]wm1$; Mhc^{10}	Female	31 ± 3
$wP[w^+Mhc^+]wm1/Y$; Mhc^{10}	Male	19 ± 3
w; $Mhc^{10}P[w^+Mhc^+]wm2$	Female	28 ± 3
w; $Mhc^{10}P[w^+Mhc^+]wm2$	Male	35 ± 3
$wP[w^+Mhc^+]wm1$	Female	15 ± 3
$wP[w^+Mhc^+]wm1/Y$	Male	14 ± 4
w; $P[w^+Mhc^+]wm2$	Female	12 ± 2
w; $P[w^+Mhc^+]wm2$	Male	16 ± 2
w; $P[w^+Mhc^+]wm3$	Female	18 ± 2
w; $P[w^+Mhc^+]wm3$	Male	17 ± 4

* Average jumping distance (see Materials and Methods) ± standard error of the mean.

Table III. Protein Accumulation in Flies with Multiple Copies of Mhc

Genotype	Sex	*Mhc/Actin88F* gene ratio*	Myosin/ actin protein ratio‡
Canton-S	Female	1.0	1.00
$Tp(2;3)osp^3/+$	Female	1.5	1.29
$wP[w^+Mhc^+]wm1/+$	Female	1.5	0.96
w; $P[w^+Mhc^+]wm2/+$	Female	1.5	1.02
w; $P[w^+Mhc^+]wm3/+$	Female	1.5	0.74
$wP[w^+Mhc^+]wm1$	Female	2.0	1.33
w; $P[w^+Mhc^+]wm2$	Female	2.0	1.43
w; $P[w^+Mhc^+]wm3$	Female	2.0	2.56
Canton-S	Male	1.0	1.00
$Tp(2;3)osp^3/+$	Male	1.5	1.20
$wP[w^+Mhc^+]wm1/Y$	Male	1.5	1.63
w; $P[w^+Mhc^+]wm2/+$	Male	1.5	1.50
w; $P[w^+Mhc^+]wm3/+$	Male	1.5	1.93
w; $P[w^+Mhc^+]wm2$	Male	2.0	2.20
w; $P[w^+Mhc^+]wm3$	Male	2.0	4.03

* In all cases flies are wild-type for the *Act88F* gene (two copies).
‡ The myosin/actin ratio as determined from scanning one-dimensional SDS-PAGE gels was normalized to 1.00 for wild-type females or wild-type males.

age compensation of inserts on the X chromosome (Tansey et al., 1987). In the past, we have used $Tp(2;3)\ osp^3$, which has a transposed copy of the genomic region including *Mhc* on the third chromosome, to rescue lethality. Using this line we were unsuccessful in obtaining rescued Mhc^1 homozygotes, despite obtaining viable homozygotes for Mhc^2, Mhc^3, and Mhc^4 (K. D. Becker, unpublished observations). We postulated that the lack of success at rescuing Mhc^1 was due to the presence of at least one additional lethal mutation on the same chromosome, which could not be rescued by the transposed copy of *Mhc*. As expected, in the crosses with the transformed genes Mhc^2 homozygotes were recovered, but not Mhc^1 homozygotes. The putative lethal mutations were therefore removed from the Mhc^1 *pr px* chromosome by recombination (see Materials and Methods), and the Mhc^1 line produced was homozygous viable in the presence of at least one copy of $P[w^+Mhc^+]wm3$. The *wm3* line clearly produces functional protein not only in the IFMs but also in all the muscles required for viability. Furthermore, Mhc^1 homozygotes with two copies of the transgene (w^{1118}; Mhc^1; $P[w^+Mhc^+]wm3$) can fly as well as wild type (Table I), indicating that sufficient levels of wild-type MHC are indeed produced from this transformed gene.

Effects of Increased Copy Number of Mhc

The relative levels of muscle proteins in the IFMs are very important for functional muscle. Not only do reductions in the levels of MHC, myosin light chain 2, actin or tropomyosin disrupt muscle function (Mogami and Hotta, 1981; Karlik and Fyrberg, 1985; Mogami et al., 1986; Warmke et al., 1988; Beall et al., 1989), but functional defects due to reductions in the levels of MHC can be partially rescued by concomitant reductions in the levels of actin (Beall et al., 1989). This restores a more normal myofibrillar structure, which allows limited flight ability. However, the effects of overabundance of muscle proteins in *Drosophila* have not been studied, other than the observation that $Tp(2;3)\ osp^3/+$ heterozygotes, which have three copies of *Mhc*, fly normally (Homyk and Emerson, 1988). We used the transformed lines to study the effects of overexpression of MHC.

One dimensional SDS-PAGE gels of the upper thoracic proteins of flies with either three or four copies of *Mhc* were scanned to determine the relative levels of myosin/actin in each of the "overexpressing" lines compared to wild type (Table III). An increase in the gene dosage of *Mhc* results in increased levels of the protein relative to actin, both in the case of three copies of *Mhc* and of four copies. This effect is more apparent in male flies than in female flies. The reason for this sex difference is not known.

In line with previous observations, the presence of three copies of *Mhc* in an otherwise wild-type background does not severely affect flight ability compared to either Canton-S or w^{1118} (Tables I and IV; Homyk and Emerson, 1988). Small differences from wild type are observed, but these are somewhat variable even between replicates of the same cross. Despite the more obvious changes in MHC accumulation in male flies, there are no consistent differences in flight ability between the sexes. At the ultrastructural level, both Canton-S and w^{1118} IFMs show normal hexagonally packed myofibrils (Fig. 5 *a*; and data not shown), and no myofibrillar defects are observed in female flies expressing three copies of *Mhc* (Fig. 5 *b*). Interestingly, some male flies with three copies of *Mhc* show a small excess of thick filaments in the IFM myofibrils (data not shown), confirming the excess MHC accumulation in males over females determined by

Figure 4. Electron micrographs of mutant and rescued IFM myofibrils. (*a*) $Mhc^1/+$; (*b*) $Mhc^1/+$; $P[w^+\ Mhc^+]wm3/+$; (*c*) $Mhc^2/+$; (*d*) $Mhc^2/+$; $P[w^+Mhc^+]wm3/+$; (*e*) Mhc^{10} homozygotes; (*f*) w;$Mhc^{10}P[w^+Mhc^+]wm2$ homozygotes. Each montage consists of three pictures: *top* (main), transverse section showing a number of myofibrils; *inset*, transverse section at higher power showing part of a single myofibril; *bottom*, longitudinal section of a single representative myofibril. Bar, 0.5 μm.

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Table IV. Flight Abilities of Flies Overexpressing Mhc

Genotype	Number tested	Flight ability (percentage)			
		Up	Horizontal	Down	Not at all
Overexpression: three copies					
Tp(2; 3)osp^3/+	109	80	5	11	5
wP[w$^+$Mhc$^+$]wm1/+ or Y	140	54	16	16	14
P[w$^+$Mhc$^+$]wm2/+	111	55	13	12	21
P[w$^+$Mhc$^+$]wm3/+	129	91	6	2	2
Overexpression: four copies					
wP[w$^+$Mhc$^+$]wm1/wP[w$^+$Mhc$^+$]wm1	69	3	0	23	74
w; P[w$^+$Mhc$^+$]wm2	106	0	0	7	93
w; P[w$^+$Mhc$^+$]wm3	118	0	0	28	72
wP[w$^+$Mhc$^+$]wm1/+ or Y; P[w$^+$Mhc$^+$]wm2/+	144	1	3	17	78
wP[w$^+$Mhc$^+$]wm1/+ or Y; P[w$^+$Mhc$^+$]wm3/+	114	9	11	40	40
P[w$^+$Mhc$^+$]wm2/+; P[w$^+$Mhc$^+$]wm3/+	105	0	8	70	22

SDS-PAGE (Table III). Thus these small increases in protein levels do not affect muscle function, but may have slight effects upon myofibril organization.

A severe effect upon flight ability is caused by the presence of four copies of *Mhc* (Table IV). This is observed in homozygotes for all three transformed lines and for all heteroallelic combinations of these, and so must be due to the presence of the transformed constructs rather than an effect of the insertional events themselves. Concomitant with this reduction in flight ability, IFM abnormalities are observed (Fig. 5 *c*): in transverse section, the myofibrils have normal hexagonally packed centers, with each hollow thick filament surrounded by six thin filaments. However, at the periphery of the myofibril the myofilaments appear to "melt". There is an overabundance of thick filaments in this area which are loosely associated with the myofibril, consistent with the overabundance of MHC in these muscles observed by SDS-PAGE. Although all the thick filaments are hollow, a characteristic of thick filaments in the IFMs, some of those at the periphery of the myofibril are smaller in diameter than the filaments in the center. Longitudinal sections of IFMs of flies containing four copies of *Mhc* confirm these observations; myofibrils with regularly spaced Z-bands are visible, however most of these show fraying of the filaments at the periphery of the myofibril.

We also jump-tested flies which overexpress *Mhc* (Table II) and compared the average jumping distance with Canton-S and w^{1118}. We noted that w^{1118} males are poor jumpers, indicating that flies wild type for *Mhc* may vary in their jumping ability, perhaps due to differences in eye color. However, flies overexpressing *Mhc* jump consistently shorter distances than w^{1118}, indicating that the mutant effects of overexpression occur in the TDT as well as in the IFMs.

Effects of Overexpression upon Viability

In order to study the effects upon muscle of further increases in *Mhc* dosage, we generated a stock that contains wild-type *Mhc* plus one copy of the second chromosome-linked transgene and one copy of the third chromosome transgene (w^{1118}; *SM1/P[w$^+$Mhc$^+$]wm2; TM2/P[w$^+$Mhc$^+$]wm3*). Flies expressing six copies of *Mhc* are not generated from this stock, indicating that six copies of *Mhc* affect muscles required for viability. Only occasionally were five copy individuals (homozygous for *P[w$^+$Mhc$^+$]wm2* and heterozygous for *P[w$^+$Mhc$^+$]wm3*) observed. This indicates that overexpression of *Mhc* can affect muscles in addition to the IFMs and the TDT.

Discussion

A major goal in our analysis of MHC in *Drosophila* is to transform flies with wild-type and mutant versions of the gene. This has proven difficult since the large plasmid size required to contain the *Mhc* gene results in drastically reduced transformation efficiency (Spradling, 1986). We show here that flies may be transformed with the entire gene together with 5′ and 3′ flanking DNA, and express high levels of wild-type protein.

This wild-type construct may now be mutated in either of two manners: to address the importance of different MHC isoforms in different muscle types, one could replace an exon which is normally expressed in nonvital muscles (such as exon 7d which is expressed in the IFMs) with an alternative exon (exon 7a, 7b, or 7c). This mutant construct, when expressed in flies, will result in an MHC isoform change in the IFMs. The biochemical and mechanical properties of these muscles can be studied to identify the contributions of different alternative exons to particular physiological properties. An alternative approach would be to make site-directed single amino acid changes to particular residues in the molecule, ideally in alternative exons expressed in muscles which are not essential for viability. This approach is made particularly useful by the recent publication of the three-dimensional structure of the myosin head (Rayment et al., 1993*a*) and a prediction of how myosin interacts with actin during the cross-bridge cycle (Rayment et al., 1993*b*). Many of the alternative exons of *Mhc* reside close to important regions of the head (Bernstein, S. I., and R. Milligan, unpublished observations) and may therefore be important in regulation of myosin activity. Both of these classes of mutant construct may be combined with the null mutants already in hand such that all of the MHC expressed in a particular line of flies derives from a mutant transformed construct.

We have previously determined the molecular lesion in a number of different randomly induced *Mhc* mutations, most of which are homozygous viable (O'Donnell et al., 1989; Collier et al., 1990; Kronert et al., 1991, 1994) but one of

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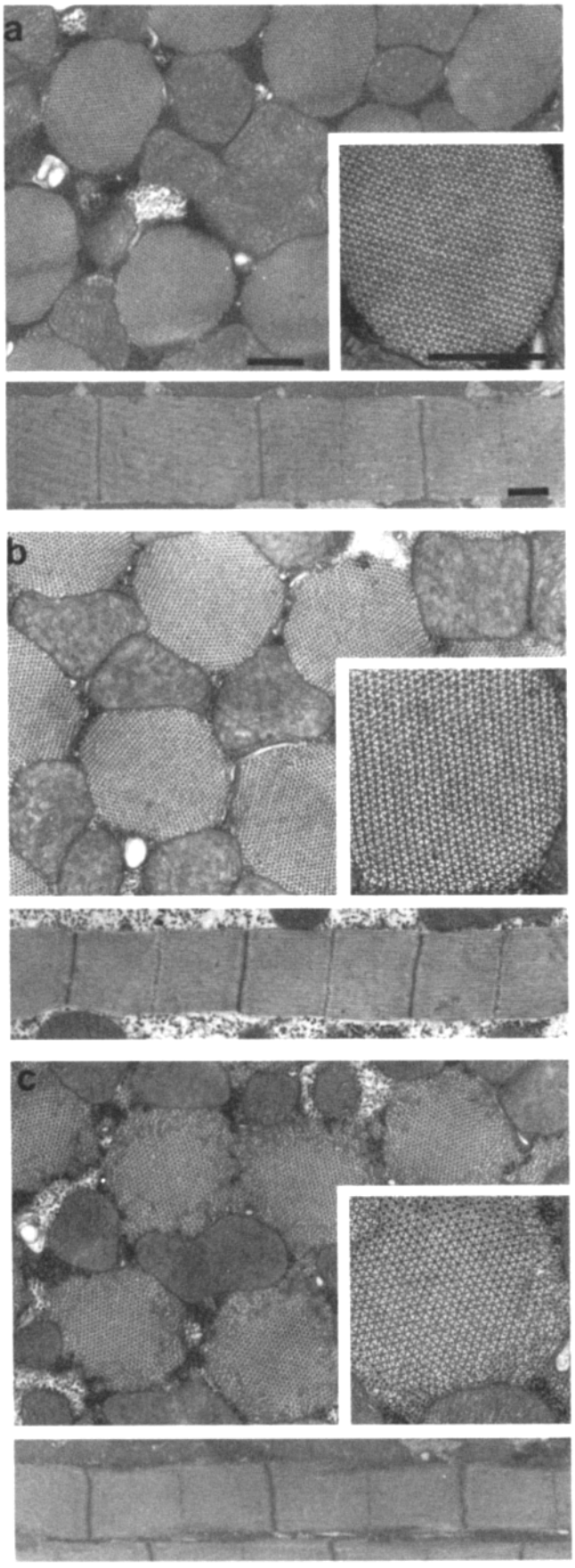

which is homozygous lethal (O'Donnell and Bernstein, 1988). Here we characterized the mutation in another lethal allele, *Mhc*^{2}, which results from the insertion of a *springer* transposable element five nucleotides upstream of alternative exon 7d. This allele is interesting since not only are levels of MHC protein reduced in both the larva and adult (Mogami et al., 1986), but MHC accumulation is completely abolished in the IFMs where exon 7d is normally included in transcripts. It is therefore a novel *Mhc* mutation which in many muscle tissues acts as an hypomorphic allele but in some muscles, in particular the IFMs, appears to be amorphic. This correlates with our electron microscopy studies which show that *Mhc*^{2}/+ IFM myofibrils are similar to those of heterozygotes for the amorphic allele *Mhc*^{1} (O'Donnell and Bernstein, 1988; and this study).

Insertional mutagenesis by a *springer* element has been reported in the *Notch* gene (Kidd and Young, 1986, the *forked* gene (Ishimaru and Saigo, 1993), and in the *Ifm(3)3* allele of the *TmI* tropomyosin gene (Karlik and Fyrberg, 1985). Both the *TmI* mutation and *Mhc*^{2} were generated by Mogami and colleagues using similar selection screens for dominant flightless mutants (Mogami and Hotta, 1981; Mogami et al., 1986). The presence of *springer* inserts in both of these genes suggests that the strain used for mutagenesis by these workers was particularly prone to mobilization of endogenous *springer* elements. Based upon this observation, it seems likely that the large insertions of DNA in *Mhc*^{3} and *Mhc*^{4} (Mogami et al., 1986) might also be *springer*-related elements.

Mhc^1/+ and *Mhc*^{2}/+ heterozygotes have IFM myofibrils in which the normal hexagonal packing of thick and thin filaments is disrupted by cracks (O'Donnell and Bernstein, 1988; and this study). This is similar to the phenotype seen in flies haploinsufficient for another thick filament structural protein, myosin light chain 2 (Warmke et al., 1988, 1992). The "cracking" phenotype seen in these mutants depends upon the presence of excess amounts of thin filament proteins, since Beall et al. (1989) showed that haploinsufficiency for both myosin and actin results in relatively normal myofibrils. This cracking effect contrasts with the phenotype of flies haploinsufficient for thin filament proteins, including actin (Beall et al., 1989) and tropomyosin (Karlik and Fyrberg, 1985; Tansey et al., 1991), where the centers of the myofibrils appear normal and are not cracked, but the peripheries show scattered and misaligned thick filaments. It appears to be the relative levels of thin filament to thick filament proteins that are important in determining whether the myofibril becomes cracked or simply disrupted at the edge. Either situation results in a flightless phenotype, presumably by affecting the contraction of the flight muscles. Our observation upon overexpression of MHC confirm this argument since we have shown that abundance of MHC relative to thin filament proteins also results in scattered and misaligned thick filaments but no cracks in the myofibril.

Figure 5. Electron micrographs of the IFM myofibrils of flies overexpressing MHC. (*a*) Canton-S wild-type; (*b*) *Tp(2;3)osp*^{3}/+ expressing three copies of *Mhc;* (*c*) *wP[w*$^+$*Mhc*$^+$*]wml* homozygotes expressing four copies of *Mhc*. Myofibrils in *a* and *b* appear normal, but those in *c* show abnormalities at their peripheries. Bar, 0.5 μm.

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The ultrastructure of IFM myofibrils is particularly sensitive to changes in gene dosage, probably due to the precise hexagonal packing of thick and thin filaments. Functionally, these changes can be detected easily as they result in a flightless phenotype. In vertebrate skeletal muscle, no mutations affecting the levels of structural proteins have been described, although similar disruptions to those we see in *Drosophila* IFMs may result since the myofilaments are also packed in an hexagonal manner. In the TDT muscle, alterations in filament packing are seen in $Mhc^1/+$ adults resulting in each thick filament being surrounded by 20 thin filaments rather than 10–11. However, no cracks in the TDT myofibrils are apparent (O'Donnell and Bernstein, 1988).

An increase in the gene copy number of *Mhc* results in an increase in the levels of MHC protein, showing that there is no mechanism by which MHC production above the level required for normal IFMs is limited. The effects of an increased gene dosage of actin have not been rigorously studied: it is not known if it results in an overabundance of the protein. A prediction, based on the above observations, is that overabundance of assembled actin protein would result in a "cracked" phenotype, similar to that observed in flies haploinsufficient for *Mhc*. However, flies containing four copies of the *Act88F* myofibrillar actin gene are not flightless (Hiromi et al., 1986). This means either that the levels of actin are regulated to prevent overabundance of the protein or that increased levels of myofibrillar actin do not affect sarcomere assembly.

The flightless phenotypes of flies homozygous for each of the *Mhc* inserts might result not from overexpression of *Mhc* but from overexpression of other genes within the ~9 kb on either side of *Mhc*. At least one gene, *TU-36B*, is known to be located in the region 3′ to *Mhc* (Levin et al., 1989). However, flies which are homozygous for both Mhc^1 and *wm3* fly well (Table I); in this situation there are still four copies of *TU-36B* and other associated genes, but only two functional copies of *Mhc*. Therefore, the flightless phenotype cannot be due to overexpression of genes other than *Mhc*. Similarly, the reduced jumping ability of $wP[w^+Mhc^+]wml$ or $w; P[w^+Mhc^+]wm2$ is not observed when the flies are also homozygous for Mhc^{10} (Table II).

We observed effects upon TDT function (a reduction in jumping ability) in flies expressing four copies of *Mhc*, indicating that mutant effects are not limited to the structurally regular IFMs but are also seen in some tubular muscles of the adult. Although we have not functionally tested other adult muscles it is apparent that overexpression of *Mhc* affects them as well: we were unable to achieve complete rescue of the flightless phenotype of Mhc^{10} homozygotes despite expressing high enough levels of protein to fully rescue IFM structure and, presumably, function. The inability to rescue flight is probably due to the fact that other muscles important for flight such as the direct flight muscles are unaffected by the Mhc^{10} mutation, but are affected by the overexpression. Given the effects upon TDT muscle function we observed in flies overexpressing MHC, and since the tubular direct flight muscles are structurally similar to the TDT, this possibility seems likely. Likewise, the flightless phenotype of flies expressing four functional copies of *Mhc* probably results not only from effects upon the IFMs but also from effects upon other muscles in the thorax which are important for flight.

Higher levels of expression of *Mhc* appear to be lethal to the organism. We have been unable to obtain any flies which contain two copies of the endogenous *Mhc* gene in addition to four transformed copies of the gene. These results indicate that altering gene dosages can severely affect muscle function, most notably in some muscles which are required for viability. The effects of overexpression of wild-type MHC have also been studied in *Caenorhabditis elegans* (Fire and Waterston, 1989). Here, extrachromosomal arrays expressing the gene which encodes MHC-B resulted in nematodes with uncoordinated locomotion. Detailed analysis of one line, in which the overexpression resulted in lethality, showed that 200–800 copies of the MHC-B gene were present, and that large amounts of MHC-B protein were deposited in blobs in the muscle cells. Our studies show that even moderate overexpression of MHC in *Drosophila* muscles can affect locomotion, and that a large amount of the overproduced protein is incorporated into thick filaments in the myofibril.

In both cases in which overexpression of MHC in muscle has been studied (our work in *Drosophila*, and that of Fire and Waterston [1989] in *C. elegans*), muscle dysfunction and abnormalities are observed. We conclude that in transgenic studies of myofibrillogenesis it is very important to confirm that effects observed in muscles are caused by the nature of the transgenic protein itself and not due simply to insufficient or excess protein. This is a particular concern in vertebrate systems where skeletal muscle structure is similar to that of *Drosophila* indirect flight muscles.

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