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Genetics

Mutations of the *Drosophila* myosin heavy-chain gene: Effects on transcription, myosin accumulation, and muscle function

(DNA insertions/transcription termination/gene dosage/dominant flightless mutant/homozygous-lethal mutant)

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ABSTRACT **Mutations of the myosin heavy-chain (MHC) gene of *Drosophila melanogaster* were identified among a group of dominant flightless and recessive lethal mutants (map position 2-52, 36A8–B1,2). One mutation is a 0.1-kilobase deletion in the 5′ region of the MHC gene and reduces MHC protein in the leg and thoracic muscles of heterozygotes to levels found in 36AC haploids. Three mutations are insertions of 8- to 10-kilobase DNA elements within the MHC gene and produce truncated MHC transcripts. Heterozygotes of these insertional mutations possess levels of MHC intermediate between those of haploids and diploids. An additional mutation has no gross alteration of the MHC gene or its RNA transcripts. Although leg and larval muscles function normally in each mutant heterozygote, indirect flight muscles are defective and possess disorganized myofibrils. Homozygous mutants die during embryonic or larval development and display abnormal muscle function prior to death. These findings provide direct genetic evidence that the MHC gene at 36B (2L) is essential for both larval and adult muscle development and function. The results are consistent with the previous molecular evidence that *Drosophila*, unlike other organisms, has only a single muscle MHC gene per haploid genome. Quantitative expression of both copies of the MHC gene is required for function of indirect flight muscle, whereas expression of a single MHC gene is sufficient for function of larval muscles and adult tubular muscles.**

Analysis of muscle mutants of the fruit fly *Drosophila melanogaster* and the small soil nematode *Caenorhabditis elegans* offers a unique approach to understanding the genetic regulation of myogenesis and muscle function (1–14). In *C. elegans*, mutations of myosin heavy-chain (MHC) (9–11), paramyosin (12), and actin (13, 14) genes have been identified, and an extensive molecular analysis has been performed. *Drosophila* is also suitable for such studies, because mutations that affect indirect flight muscle function and myofibrillar organization can readily be isolated and do not affect viability. Mutations have been identified in the flight muscle-specific actin gene at 88F (3R) (5–7). In contrast to the actin genes, which constitute a family of genes that are differentially expressed in a stage- and tissue-specific manner (15, 16), the *Drosophila* MHC gene at 36B (2L) is single-copy in the haploid genome and is expressed in all muscle types (17, 18).

Previously, we have shown that haploidy of the MHC gene region at 36AC (2L) results in a reduction of MHC in adult thoracic muscle and tubular leg muscle and in larvae; only indirect flight muscle function is abnormal in these haploids (17). These findings suggested that flight muscle function is more sensitive than that of other muscle types to the level of MHC gene expression. On this basis, we reasoned that heterozygous mutations that reduce the level of MHC expression would selectively affect flight muscle function, whereas such mutations, when homozygous, should affect the function of all muscle types and may cause embryonic lethality.

Dominant mutations affecting indirect flight muscle function have been mapped to the 36AC chromosomal region, near the site of the MHC gene (2, 17, 19). In this study, we have used genetic and molecular approaches to identify five of these as MHC gene mutations. As predicted, two copies of the normal MHC gene are required for the development of functional indirect flight muscle, whereas a single copy is sufficient for function of larval and adult leg muscles. Homozygous mutants die during embryonic or larval development, providing additional evidence that the single-copy MHC gene at 36B (2L) encodes the predominant MHC protein of larval and adult muscles (17, 18).

MATERIALS AND METHODS

***Drosophila* Strains and Genetic Manipulations.** *D. melanogaster* Canton-S (20) was used as a standard strain. Fifteen second-chromosomal dominant flightless mutants were examined. One mutant (Bashed) was reported by Grell (19). The other mutants, including *Ifm(2)3* and four *Mhc* (Myosin heavy chain) mutants, were isolated by K.M. and Y. Hotta (ref. 2 and unpublished work) by testing the flight ability of progeny of normal females mated to normal males fed with the mutagen ethyl methanesulfonate.

Overlapping deficiencies and duplications between 34B and 40A on the salivary gland chromosome map were generated by the segmental aneuploidy technique (21). Chromosomal rearrangements within this region were also examined (22–25). The following stocks are referred to in this article: B214 and A62 (ref. 21, *Y;2* translocation stocks with breakpoints, according to M. Ashburner, at 36A1,2-6,7 and 36C1,2, respectively); *Df(2L)36AC*, a deficiency stock made by the cross of B214 and A62; *Df(2L)H20* = *Df(2L)36A6, 7;36F1,2*, and *Df(2L)H68* = *Df(2L)36B1,2;37B1* (cytology according to R. Steward). To determine the cytological location of the *Mhc* mutations, *Mhc/SM1* flies were crossed to the deficiency stocks balanced over *SM1* or *CyO* (see ref. 20 for these balancer chromosomes).

Genetic mapping experiments and examination of indirect flight muscle myofibrils were performed according to ref. 2.

Lethal-Period Determination. Each mutant line was crossed with wild-type (Canton-S strain) flies. Males and females heterozygous for the same *Mhc* mutation (*Mhc*/+) were subsequently crossed to each other. Embryos were

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Abbreviations: MHC, myosin heavy chain; kb, kilobase(s).

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collected, dechorionated by treating with 50% Clorox for 1 min, and observed until hatching. Larvae were transferred to food vials and allowed to pupate and emerge. Approximately 100 embryos were examined for each strain.

Nucleic Acid Analysis. DNA samples were extracted from normal (Canton-S strain) and dominant flightless mutants according to R. Lifton (cited in ref. 26). After digestion with each of four restriction enzymes and electrophoresis, genomic blots were hybridized with the cloned *Drosophila* MHC gene or its various subfragments (17). Rearrangements were deduced from the abnormal DNA bands.

To prepare RNA samples, pupae were collected within 1 day after eye pigment deposition. Pupae were ground in a 55-ml Teflon-on-glass tissue grinder in a buffer containing guanidinium isothiocyanate, and RNA was isolated by CsCl gradient centrifugation (27). Poly(A)$^+$ RNA was selected by chromatography of total RNA on oligo(dT)-cellulose columns (28). RNA samples were electrophoresed in a 0.8% agarose gel containing formaldehyde (28), blotted onto nitrocellulose, and probed with a nick-translated probe containing the 5′ region of the MHC gene.

Protein Analysis. Three female thoraces were homogenized in 70 μl of sample loading buffer (29). Thirty-five microliters of the final volume was loaded onto a 10% polyacrylamide gel containing $NaDodSO_4$ (29). For leg-protein analysis, legs from six females were homogenized in 45 μl of buffer and the entire sample was loaded on an 11% gel. After electrophoresis, the gels were stained with Coomassie brilliant blue and the lanes scanned with a laser densitometer. To correct for variations in amount of protein loaded, the observed MHC content was divided by the value obtained for two major thoracic proteins (M_r 45,000 and 53,000) or a single leg protein (M_r 45,000). Accumulation of these proteins is not affected by the mutations (data not shown). MHC content was then calculated as a percentage of wild-type levels.

RESULTS

Genetic and Morphological Analysis of *Mhc* Mutants. To identify MHC mutants, we screened 15 second-chromosomal dominant flightless mutants by genomic Southern blotting. MHC gene rearrangements were found in four of these mutants (*Mhc*^{1}, *Mhc*^{2}, *Mhc*^{3}, and *Mhc*^{4}; see below).

The four *Mhc* mutants were isolated as dominant flightless offspring of flies treated with the mutagen ethyl methanesulfonate. Their flightless phenotypes map within the chromosome-2 cluster of indirect flight muscle mutations (genetic map position 52, ref. 2). Their indirect flight muscles have disrupted myofibrils (Fig. 1). To define precisely the cytological location of these mutations, we examined flight muscle function of flies with overlapping deficiencies between regions 34B and 40A on the salivary gland chromosome map (21–25). Deficiency heterozygotes *Df(2L)H20*/+ do not fly and have disrupted myofibrils in their indirect flight

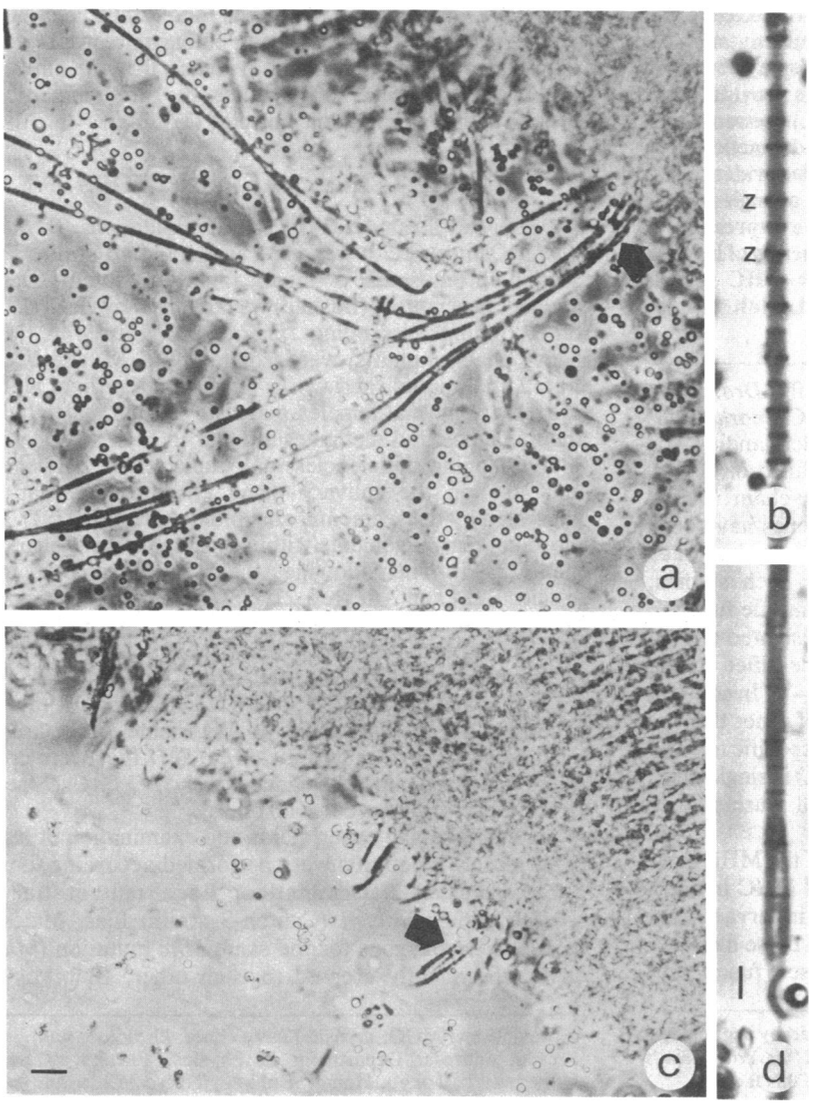

FIG. 1. Phase-contrast micrographs of indirect flight muscle myofibrils of normal flies (*a* and *b*) and *Mhc*^{1} heterozygotes (*c* and *d*). Thoraces were dissected in Ringer solution and observed with a phase-contrast microscope (2). In a normal thorax at a low magnification (*a*), abundant and straight myofibrils protrude from cut ends of indirect flight muscle cells (arrow). At a higher magnification (*b*), myofibrils show a regular banding pattern. In *Mhc*^{1}/+ flies (*c*), the protruding myofibrils are relatively short (arrow), probably because they are mechanically more fragile. At a higher magnification (*d*), spaces are frequently observed at the center of myofibrils. Abnormalities were also observed with an electron microscope (data not shown). *Mhc*^{2}, *Mhc*^{3}, *Mhc*^{4}, and 36AB-deficiency heterozygotes show similar abnormalities. Z, Z-band. Scale bar in *c* (also for *a*) = 10 μm; bar in *d* (also for *b*) = 2 μm. See ref. 2 for myofibrils of *Ifm(2)3* flies.

muscles, whereas *Df(2L)H68*/+ heterozygotes are wild-type for both phenotypes. Based upon the breakpoints of these deficiencies (see *Materials and Methods*), these results define the haplo-insufficient region for flight and myofibril formation as 36A8-B1,2. Further, using the duplication stock made by crossing the two *Y;2* translocations B214 and A62 (21), we have generated flies having two normal alleles and a mutant allele ($Mhc^+/Mhc^+/Mhc$). Both the dominant flightless phenotype and the disrupted myofibril phenotype of Mhc^1, Mhc^2, Mhc^3, and Mhc^4 were rescued in these aneuploid flies (at least 30 flies were examined for each strain). These results indicate that the *Mhc* mutations map to the cytological location of the MHC gene as defined by *in situ* hybridization (17, 18).

Each of the *Mhc* mutants possesses a recessive lethal phenotype within the same complementation group. To date, their homozygous lethality has been inseparable from their dominant flightless phenotype, suggesting that the two phenotypes result from the same mutation. By crossing the *Mhc* mutants with the deficiency stocks, the lethal phenotype was also mapped to the same 9-band region (Table 1).

One previously characterized (2) dominant flightless mutation, *Ifm(2)3*, was found to be an allele of the *Mhc* mutations. It is a recessive lethal mutation that is not viable in combination with the other *Mhc* alleles. As expected, the lethal phenotype of this mutant is located in 36A8-B1,2 [lethal over *Df(2L)H20* but not over *Df(2L)H68*, see Table 1]. However, the disrupted myofibrils and flightless phenotypes are not rescued by a duplication of 36AC, nor by an even larger overlapping duplication. *Ifm(2)3*/+ flies have severe thorax-indentation and/or vertical wing-position phenotypes, whereas the *Mhc* mutants and deficiency heterozygotes do not. Although MHC protein levels are reduced in *Ifm(2)3*/+ flies, no abnormal MHC DNA, RNA, or protein was detected (see below). It is therefore likely that *Ifm(2)3* is a missense mutation. Missense mutations that affect MHC stability and function have been identified in *C. elegans* (11).

Lethal Periods of Homozygous *Mhc* Mutants. Homozygous *Mhc* mutants die during late embryonic or larval development (Table 1). Mhc^1 and Mhc^4 are embryonic lethals. Both mutants appear to undergo normal embryonic development; however, the former shows no muscular movement, and the latter moves but is unable to break through the vitelline membrane. Mhc^1/Mhc^4 individuals die at the embryonic stage (data not shown), indicating that the effective lethal phase for Mhc^1 or Mhc^4 homozygotes is not caused by a mutation outside the *Mhc* gene. The Mhc^+ gene therefore is required for normal larval development.

Molecular Identification of MHC Mutations. Blots of genomic DNA from *Mhc* mutant heterozygotes were hybridized with the cloned MHC gene (17). One or two unexpected restriction fragment(s) were detected in addition to the expected fragments. More detailed analysis of mutant DNA, using smaller probes and four restriction enzymes, allowed us to map the location of these unexpected fragments within the MHC gene (Fig. 2).

Mhc^1 has a 0.1-kilobase (kb) deletion located within the first exon of the gene, as determined by the R-loop and nuclease S1 analyses of Rozek and Davidson (18). Mhc^2 and Mhc^3 have 10-kb insertions at slightly different positions near or within the third intron. Mhc^4 has a 9-kb insertion in the fourth intron. *Ifm(2)3* has no gross alteration of the MHC gene.

To examine whether the mutant MHC gene rearrangements disrupt production of MHC transcripts, we hybridized radiolabeled MHC DNA to gel blots of RNA from wild-type and mutant pupae (Fig. 3). Wild-type MHC genes produce 7.2-, 8.0-, and 8.6-kb transcripts in pupae (lane 6; ref. 18 and S.I.B., C. J. Hansen, K. D. Becker, D. R. Wassenberg, E. S. Roche, J. J. Donady, and C.P.E., unpublished data). The small deletion in the Mhc^1 gene does not cause detectable differences in MHC transcript sizes. However, each of three mutant heterozygotes carrying DNA insertions ($Mhc^2/+$, $Mhc^3/+$, and $Mhc^4/+$) produces an aberrantly small MHC transcript in pupae in addition to the three normal-sized transcripts. The aberrant transcripts are also expressed during larval muscle differentiation (data not shown).

The accumulation of thoracic MHC protein is also disrupted in heterozygous MHC mutants (*Mhc*/+). The thorax of flies consists largely of indirect flight muscle and other muscle fibers (30). No aberrant proteins were detected in thoraces of mutants (data not shown). However, MHC protein in $Mhc^1/+$ thorax is reduced to the level found in 36AC haploids [*Df(2L)36AC*/+]. Heterozygotes of other alleles possess levels of MHC intermediate between those of haploids and diploids (Table 1). Levels of MHC protein in legs of mutant heterozygotes and those carrying 36AC heterozygous deficiencies are similarly reduced (Table 1), although no abnormalities in leg function were evident.

DISCUSSION

Molecular Defects of *Mhc* Mutants. In this report, we have identified and characterized mutations in the MHC gene of *D.*

Table 1. Summary of mutant characters

	Cross with deficiencies*		DNA rearrangements†	Non-wild-type transcripts‡	MHC protein, % wild type§		% lethality at each stage¶		
	H20	*H68*			Thorax	Legs	Embryos	Larvae	Pupae
Canton-S (normal)			—	—	100	100	7	7	7
Mhc^1	0/232	73/481	0.1-kb deletion	—	64 ± 9	62 ± 4	*24*	9	5
Mhc^2	0/247	175/586	10-kb insertion	3.7 kb	77 ± 7	68 ± 7	3	*39*	1
Mhc^3	0/235	102/465	10-kb insertion	4.1 kb	89 ± 3	78 ± 13	1	*29*	1
Mhc^4	0/222	265/1017	9-kb insertion	2.4 kb	78 ± 5	70 ± 11	*23*	13	6
Ifm(2)3	0/249	52/193	—(point mutation?)	—	79 ± 3	77 ± 9	5	*29*	5

—, No difference detected.

*An example of results that indicate that the *Mhc* mutations are located at 36AB. Of the offspring of *Mhc/CyO* flies mated to flies heterozygous for either *Df(2L)H20* [= *Df(2L)36A6,7;36F1,2*] or *Df(2L)H68* [= *Df(2L)36B1,2;37B1*], 33% will have the *Mhc* mutation and the deficiency chromosome. The number of *Mhc/Df* flies recovered (numerator) compared to total number examined (denominator) indicates that the lethality associated with the *Mhc* mutations maps in the interval 36A8–36B1,2 (see text).

†See Fig. 2.

‡See Fig. 3.

§Values shown are the means and standard deviations obtained from three independent experiments. MHC protein in thorax and legs of *Df(2L)36AC*/+ were 62 ± 3% and 61 ± 11% of wild type, respectively.

¶Males and females heterozygous for the same mutation (*Mhc*/+) were crossed. Numbers of embryos, larvae, pupae, and adults were counted, and the lethality at each stage was calculated. Italics indicate the effective lethal phase. Numbers of unfertilized eggs are not included.

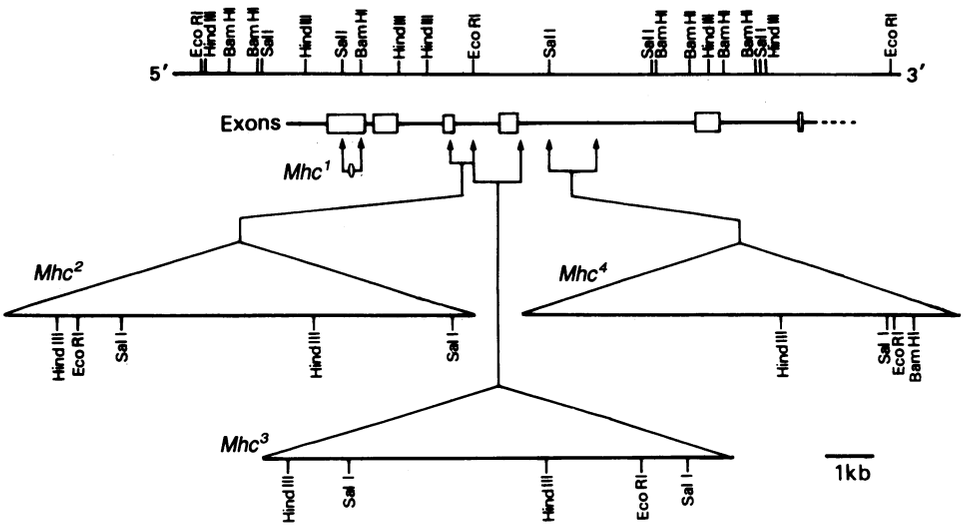

FIG. 2. DNA restriction maps of normal and *Mhc* mutant flies. The top line is a restriction map of the 5' portion of the normal *Drosophila* MHC gene (17, 18). Below the map, exons of the gene are indicated by open boxes (18). DNA aberrations of *Mhc* mutants are shown either by parentheses (deletion, Mhc^1) or by triangles (insertion, Mhc^2, Mhc^3, Mhc^4). The limits of uncertainty about the positions of rearrangements are indicated by vertical arrows. The Mhc^1 mutant has a deletion of ≈0.1 kb at the 5' end of the gene. The mutants Mhc^2 and Mhc^3 have similar insertions (10 kb) at slightly different positions. They have at least one *Eco*RI site, two *Hin*dIII sites, two *Sal* I sites, and no *Bam*HI sites. The total length and the relative positions of the *Hin*dIII and *Sal* I sites are identical in both insertions. The insertions may be related mobile genetic elements. The insertion in Mhc^4 has at least one *Eco*RI, one *Hin*dIII, one *Sal* I, and one *Bam*HI site. Because the probes hybridize only with the flanking regions of the insertions, and because such regions are sometimes too short to be detected, our maps of the insertions may not be complete.

melanogaster. The molecular and developmental alterations caused by these mutations, such as smaller MHC transcripts, reduced MHC protein content, disrupted myofibrils, and embryonic lethality, are interpretable based on the observed DNA lesions in the MHC gene. Mhc^1 is a small first-exon deletion that probably introduces a translational stop codon causing premature translation termination. Leg and thoracic

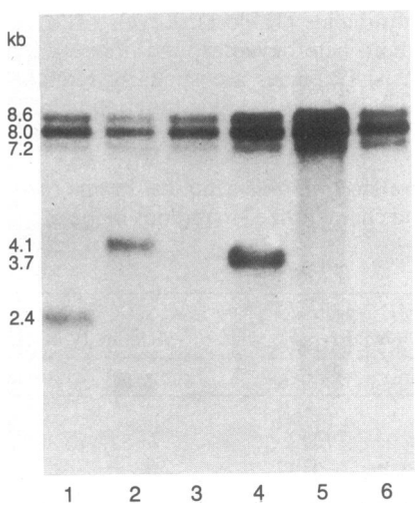

FIG. 3. Pupal MHC RNA accumulation in normal flies (Canton-S) and *Mhc*/+ heterozygotes, as shown by autoradiography of an RNA gel blot probed with a nick-translated MHC gene. Each lane contained 1 μg of poly(A)$^+$ RNA. Normal pupal MHC transcripts (lane 6) are 7.2-, 8.0-, and 8.6-kb long (ref. 18 and unpublished data). Mutant Mhc^1 (lane 3), which has a 0.1-kb deletion in the first exon, shows the normal RNA pattern. Note, however, that transcripts with this small deletion may not be resolved from wild-type transcripts. Mutant heterozygotes of Mhc^2 (lane 4), Mhc^3 (lane 2), and Mhc^4 (lane 1) produce aberrant MHC RNAs of the indicated lengths. The pattern of MHC RNA accumulation in *Ifm(2)3*/+ (lane 5) is identical to the wild-type pattern. Since no DNA rearrangements have been detected in this mutant, it may be a point mutation. In all cases poly(A)$^+$ and total RNA yielded identical results.

proteins of Mhc^1/+ heterozygotes contain haploid levels of MHC. DNA insertions in the Mhc^2, Mhc^3, and Mhc^4 genes likely possess transcription termination and polyadenylylation signals, and the different lengths of each abnormal transcript result from the location of these signals within the insertion, as well as the location of the insertion within the MHC gene (P.T.O. and S.I.B., unpublished data; see refs. 8, 31, and 32 for other insertional mutations). If N-terminal proteins are synthesized from these truncated transcripts, they must be unstable (see ref. 11 concerning the stability of mutant MHC in the nematode) or very small, since aberrant proteins have not been detected by gel electrophoresis. Of particular interest is the finding that the MHC protein contents of Mhc^2/+, Mhc^3/+, and Mhc^4/+ heterozygotes are not as low as those of 36AC haplo-deficient or Mhc^1/+ flies, suggesting that the mutant alleles with DNA insertions produce some functional MHC mRNA. Since these DNA insertions are probably within introns (see Fig. 2), this indicates that transcription is not always terminated within the insertion and that some of the abnormally large MHC transcripts from these mutant alleles are properly spliced, leading to the production of MHC mRNA. Levis *et al.* (31) reported that DNA insertions at the white (*w*) locus apparently cause a "leaky" phenotype by this mechanism. Assuming that some of the MHC protein produced from these mutant genes is normal, it is remarkable that flight muscle with nearly 90% normal levels of MHC protein is nonfunctional. This indicates that flight muscle function is exquisitely sensitive to MHC protein level.

The DNA aberrations observed in these *Mhc* mutants were unexpected, since the mutagen ethyl methanesulfonate causes point mutations in prokaryotes (33). In addition to the three insertional mutants reported here, an ethyl methanesulfonate-induced mutation in the *Drosophila* tropomyosin gene, *Ifm(3)3*, has a DNA insertion of 8 kb (8). In *Drosophila*, hybrid dysgenesis is caused by insertion of mobile elements (32). However, our original scheme of mutant isolation (2) attempted to minimize the possibility of such dysgenic processes. There may be a mechanism in *Drosophila* that mobilizes DNA elements when DNA bases are alkylated.

Alternatively, these elements may have inserted spontaneously. The latter possibility seems unlikely because these dominant mutants were recovered as 0.026% of progeny of ethyl methanesulfonate-treated flies (Y. Hotta and H. Hata, personal communication), a level well above the spontaneous mutation rate.

Muscle Dysfunction and Lethality of *Mhc* Mutations. *Mhc* mutations have a dominant flightless phenotype but also are lethal in the homozygous state. Mhc^1 homozygotes die before hatching without any muscular movement. The embryonic lethality of this mutant adds genetic support to the earlier conclusion that *Drosophila* has a single MHC gene per haploid genome, rather than a family of genes encoding muscle MHC (17, 18). This MHC gene is expressed and required for both larval and adult muscle function. Among organisms studied to date, *Drosophila* is unique in having only a single muscle MHC gene. In vertebrates such as chicken (34, 35), mouse (36, 37), rat (38, 39), and human (40) and in the invertebrate *C. elegans* (41), MHC is encoded by families of related genes with divergent coding potentials. Specific family members are selectively expressed in different muscle types at different stages of development. Although *Drosophila* has only a single muscle MHC gene, this gene produces at least four different MHC transcripts that differentially accumulate during development. Alternative patterns of RNA splicing produce transcripts that encode MHCs with different C-termini (ref. 18 and unpublished results).

Our genetic and molecular evidence that *Drosophila* has a single MHC gene also has important implications for understanding the mechanisms regulating muscle gene expression during the development of the specialized muscle types of this organism. The MHC gene is transcriptionally activated in all muscle cell types, in contrast to the actin multigene family whose members are activated in different muscle types (15, 16). Thus, actin genes must have evolved transcriptional regulatory mechanisms distinct from those controlling the MHC gene, in order to exhibit their stage- and tissue-specific expression.

Finally, our data on MHC mutations support the idea of Mogami and Hotta, who hypothesized that indirect flight muscle function would be sensitive to the dosage of myofibrillar-protein gene expression and that mutations in these genes would cause a dominant flightless phenotype (1, 2). The development of indirect flight muscle is unique in its dependence on the dosage of the MHC gene, indicating that the assembly of the highly structured myofibrils of this muscle is more easily disrupted than in other muscle types that have less-ordered myofibrils. Dominant hypomorphic mutations in an actin gene (6, 7) and a tropomyosin gene (8) also disrupt flight muscle function, indicating a sensitivity to the dosage of these myofibrillar-protein genes as well. However, unlike the MHC gene mutations, the actin and tropomyosin gene mutations affect only flight muscle-specific transcripts. Thus these mutations, even when homozygous, do not disrupt function in other muscle types. Other dominant indirect flight muscle mutations are closely linked to the *Mhc* mutations (refs. 2 and 19; K.M., unpublished data). Chromosomal "walking" (26) will be useful to identify these mutant muscle genes and to examine their function in flight muscle and the significance of the clustering of muscle genes at the 36B (2L) locus.

Note Added in Proof. At least one additional exon is located 5′ of the first exon shown in Fig. 2 (D. R. Wassenberg and S.I.B., unpublished data).

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