Co-Localization to Chromosome Bands 99E1-3 of the *Drosophila melanogaster* Myosin Light Chain-2 Gene and a Haplo-Insufficient Locus That Affects Flight Behavior

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ABSTRACT

Using overlapping synthetic deficiencies, we find that a haplo-insufficient locus affecting flight behavior and the myosin light chain-2 gene co-map to the *Drosophila melanogaster* polytene chromosome interval 99D9-E1 to 99E2-3. From screening over 9000 EMS-treated chromosomes, we obtained alleles of two complementation groups that map to this same interval. One of these complementation groups, *Ifm(3)99Eb*, exhibits dominant flightless behavior; thus, flightless behavior of the deficiency is in all likelihood due to hemizygosity of this single locus. Rescue of flightless behavior by a duplication indicates that the single allele, *E38*, of the *Ifm(3)99Eb* complementation group is a hypomorph. Based upon its map position and a reduction in concentration of myosin light chain-2 mRNA in heterozygotes, we propose that $Ifm(3)99Eb^{E38}$ is a mutant allele of the myosin light chain-2 gene. Our genetic analysis also resulted in the identification of four dominant flightless alleles of an unlinked locus, *l(3)nc99Eb*, that exhibits dominant lethal synergism with *Ifm(3)99Eb*.

MYOSIN is a 450,000-dalton multi-subunit protein consisting of two myosin heavy chain (MHC) subunits and two chemically distinct myosin light chains, myosin light chain-2 (MLC-2, the regulatory light chain) and myosin alkali light chain (MLC-ALK, the essential light chain). Myosin binds reversibly to actin and possesses a Mg^{2+}-ATPase activity. This enzymatic activity provides the energy required for motility events, not only organismic motility provided by muscular contraction but cellular motility via ameoboid movement (reviewed by KORN 1978). There are two independent systems that regulate the activity of the Mg^{2+}-ATPase, a thin filament system (troponin-tropomyosin mediated) and a myosin linked system (MLC-2 mediated). Phosphorylation of MLC-2 has been shown to be necessary for or has been correlated with the following events: the development of rigor in vertebrate smooth muscle, regulating the assembly of myosin monomers, altering the conformation of myosin dimers, increasing the calcium sensitivity of frog skinned muscle fibers, and modifying the Mg^{2+}-ATPase activity in skeletal muscle independent of thin filament control (SELLERS and ADELSTEIN 1987). Scallop MLC-2, which is not phosphorylated, directly influences the structure and function of myosin heads. Upon physical removal of MLC-2 from the myosin heads or upon Ca^{2+} binding by MLC-2, the myosin heads alter their configuration resulting in an increase in Mg^{2+}-ATPase activity and the development of tension (VIBERT and CRAIG 1985). The mechanism by which phosphorylation of MLC-2 or calcium binding regulates the myosin-linked Mg^{2+}-ATPase has not been fully defined. The sophisticated genetics of Drosophila provides a particularly powerful approach with which to investigate structure-function relationships of the MLC-2 protein. Through the isolation and subsequent characterization of mutations in the MLC-2 gene we can test directly the effects that specific structural changes have on the myosin-linked Mg^{2+}-ATPase.

We have directed our efforts to defining the role of the Drosophila MLC-2, the regulatory light chain of Drosophila, as it pertains to the structure and function of the indirect flight muscle (IFM) myofibril. MLC-2 is a single copy gene that maps to polytene chromosome bands 99E1-3 (PARKER, FALKENTHAL and DAVIDSON 1985; TOFFENETTI, MISCHKE and PARDUE 1987). Two transcripts of 1.1 and 1.4 kb that differ only in the site of polyadenylation encode a single polypeptide that is expressed in both tubular and fibrillar muscles. Different domains in the MLC-2 protein have been identified by amino acid sequence comparisons. These include an apparently functional calcium binding domain and a phosphorylation site (PARKER, FALKENTHAL and DAVIDSON 1985) which is phosphorylated *in vivo* (M. GRAHAM, M. CHUN and S. FALKENTHAL, unpublished results).

In this communication we describe a genetic characterization of the polytene chromosome interval 99D3 to 99E2-3 which includes the MLC-2 gene. We demonstrate that the distal breakpoint of the synthetic deficiency used for a series of genetic screens is within the first intron of the MLC-2 gene. Flies carrying this deficiency exhibit dominant flightless behavior that is

TABLE 1

Chromosome rearrangements

Stock	Segment duplicated	Segment deleted	Reference[a]
Dp(3;1)124P; Df(3R)B81,P[rp49]/TM3		99D3 to 99E2-3[b]	1, 2
Dp(3;1)67A; Df(3R)B81,P[rp49]/TM3		99D3 to 99D9-E1[b]	1, 2
Dp(3;1)R10; Df(3R)B81,P[rp49]/TM3		99D3 to 99D6-9[b]	1, 2
Dp(3;1)67A	99D9-E1 to 100F		2, 3
Dp(3;1)124P	99E2-3 to 100F[b]		3
Dp(3;1)R14	99D1-2 to 100F[b]		2, 3

The Dp(3;1) stocks are X-ray-induced deficiencies of Dp(3;1)B152 which contains the terminal portion of distal 3R from 98F14 appended to the right arm of the *X* chromosome (FRISARDI and MACINTYRE, 1984).

[a] 1, KONGSUWAN *et al.* (1985); 2, KONGSUWAN, DELLAVALLE and MERRIAM (1986); 3, FRISARDI and MACINTYRE (1984).

[b] Changed by the authors.

the result of hemizygosity for only one complementation group, *Ifm(3)99Eb*. We propose that *Ifm(3)99Eb* encodes the MLC-2 protein. Additionally, we have identified four alleles of a distinct noncomplementing locus. Alleles of this locus confer dominant flightless behavior, fail to complement a synthetic deficiency for the region containing the MLC-2 gene and show allele specific complementation behavior with the putative MLC-2 allele, *Ifm(3)99Eb^{E38}*.

MATERIALS AND METHODS

Fly stocks and culture conditions: The synthetic deficiencies used in this analysis are of the genotype: *Dp(3;1)/X* or *Y; T(Y;3)3^{P}Y^{D}*/balancer, where *T(Y;3)3^{P}Y^{D}* represents the deficiency segregant of the reciprocal *(Y;3)* translocation. These synthetic deficiencies and a number of *Dp(3;1)* stocks are listed in Table 1. The construction of these deficiencies has been described previously (KONGSUWAN, DELLAVALLE and MERRIAM 1986). All synthetic deficiencies uncover *M(3)99D* which is a strong *Minute* characterized by small bristles, slightly abnormal wings and a 2–3-day delay in eclosion compared to wild type (KONGSUWAN, DELLAVALLE and MERRIAM 1986). This phenotype is due to haploidy for the *rp49* gene and can be rescued by transformation with the wild type *rp49* gene (KONGSUWAN *et al.* 1985). To facilitate use of these synthetic deficiencies in genetic screens, a transformed copy of the *rp49* gene that inserted at 84F was recombined onto the deficiency segregant of the *T(Y;3)B81* chromosome. The synthetic deficiencies and *Dp(3;1)* stocks were generously provided by JOHN MERRIAM.

The *C10* mutation was originally isolated in a hybrid dysgenic screen for embryonic recessive lethal mutations by ROGER KARESS on a *red e* chromosome. Hybridization *in situ* and whole genome Southern analysis utilizing *P* element hybridization probes indicated that no *P* element sequences are present in this stock (data not shown). *ca E38* was obtained in the laboratory of JOHN MERRIAM from a lethal screen of EMS mutagenesized *ca* chromosomes using *Df-B81Dp67A* as a tester chromosome. *E38-13* is a derivative of the *ca E38* chromosome in which the *E38* mutation was recombined onto a Canton-S third chromosome. Both *C10* and *ca E38* were generously provided by JOHN MERRIAM.

Third chromosome balancers used are *In(3LR)TM3, ri p^{p} sep su(Hw)^{2} Sb bx^{34e} e* and *In(3LR)TM6B, Hu e Tb ca* (CRAYMER 1984), and will be referred to as *TM3* and *TM6B*, respectively. Other mutations and balancers used are described in LINDSLEY and GRELL (1968) or LINDSLEY and ZIMM (1985, 1987).

Unless otherwise indicated, all fly stocks and crosses were maintained at 22° or room temperature on agar-cornmeal based media (LEWIS 1960). For cytological analysis of salivary gland chromosomes, larvae were grown at 18° at low density on agar-cornmeal based media onto which live baker's yeast was seeded.

Isolation of recessive lethal mutations in the 99D3 to 99E2-3 region: Males, aged 1–2 days, were mutagenized with 0.025 M EMS (Sigma) according to the method of LEWIS and BACHER (1968). Males for the S screen were of the genotype *e ro ca*, while Canton-S males were used for the J, L and SF screens. Following mutagenesis groups of ten males were mated to 20 virgin females of the genotype *ru h th st cu sr e^{s} Pr ca/TM6B* in half-pint bottles for 4 days, then the males were cleared and the females were transferred to new bottles. F_1 progeny (*e^{s} ro ca*/TM6B* males and females, or *e^{s} ro ca*/ru h th st cu sr e^{s} Pr ca* males) were mated individually to *DfB81Dp124P/TM3*. The resulting F_2 progeny were scored for the absence of the *e^{s} ro ca*/DfB81Dp124P* progeny class. If this class was lethal, a stock was established by sib mating the *e^{s} ro ca*/TM3* F_2 progeny. Mutagenesis of Canton-S chromosomes was handled similarly. If a particular stock showed reduced viability (less than 10%) it was analyzed for conditional lethality at 18° or 29°.

Complementation analyses: To determine complementation groups among the recessive lethal mutations, *inter se* crosses were performed between all mutants that mapped either within the 99D6 to 99D9-E1 subinterval or the 99D9-E1 to 99E2-3 subinterval. Because all recovered mutants were balanced over *TM3*, the absence of any Sb^{+} flies was used as the criterion for failure to complement. Approximately 100 progeny were scored for each cross. Crosses involving temperature sensitive alleles were done at 29°.

Recombinant mapping and flight testing: To determine if the lethal mutations mapped to the region defined by the *DfB81Dp124P* deficiency or were located elsewhere, recombination mapping was performed between one allele of each complementation group (*l(3)*a. . .i/*TM3*) and *J1/TM3*. *J1* was selected as the lethal to which all other lethals would be mapped, because it was the strongest allele recovered for complementation group *l(3)99Ea*, and all alleles of this complementation group exhibited normal complementation behavior. As outlined in Figure 1, males (*l(3)*a. . .i/*TM3*) were mated to virgin females (*J1/TM3*); F_1 virgin females (*l(3)*a. . .i/*J1*) were mated to *DfB81Dp124P/TM3* males. Approximately 500 progeny were scored for the presence of the Sb^{+} (*l(3)*a. . .i^{+} *J1^{+}/DfB81Dp124P*) recombinant class.

To determine if the flightless behavior of the mutant stocks could be rescued by increasing the wild type gene dosage, the duplication *Dp(3;1)R14* was crossed into each mutant stock (as outlined in Figure 2), and flies of the

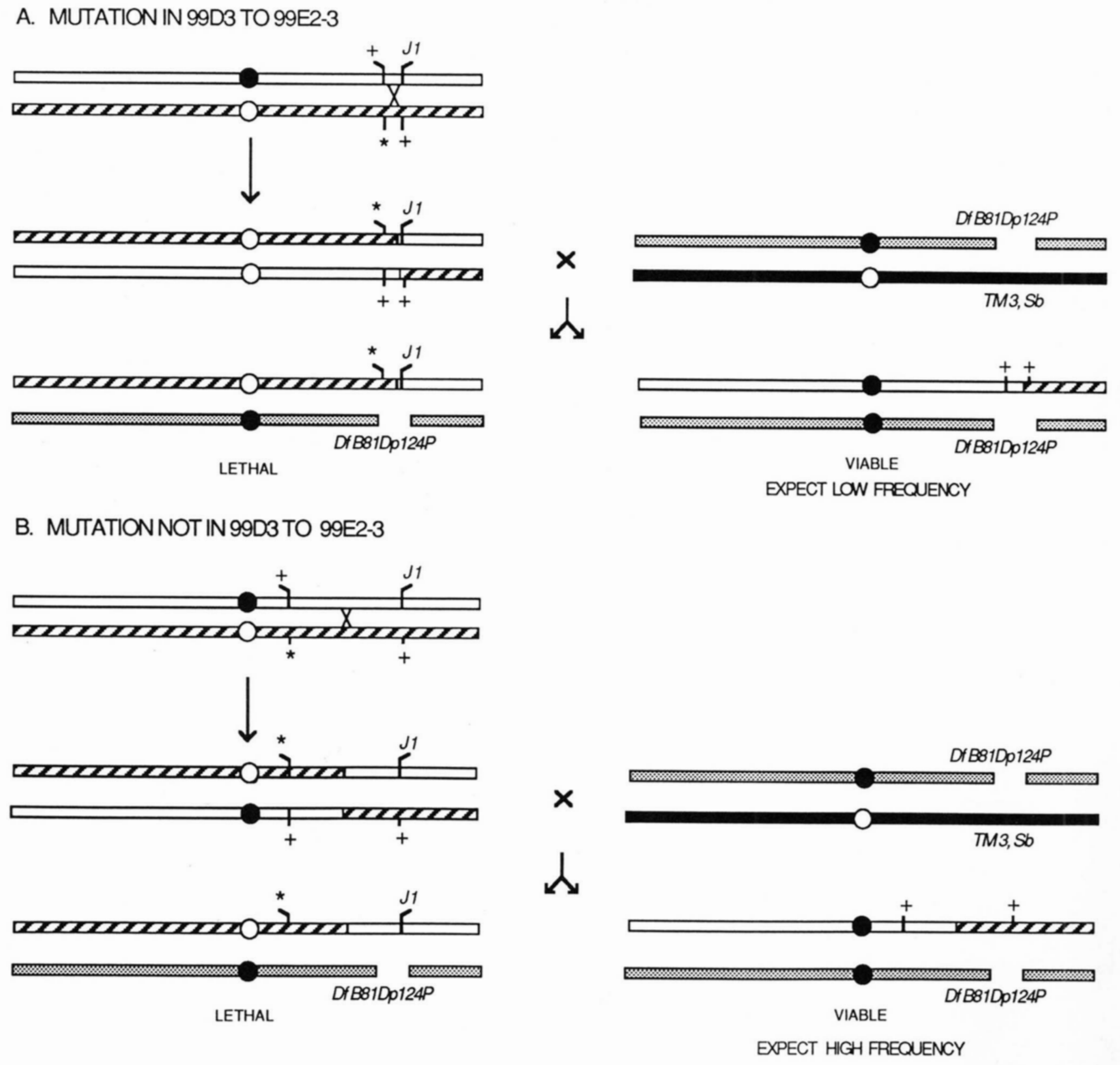

FIGURE 1.—Mapping strategy used to determine if lethal mutations map to the 99D3 to 99E2-3 region. Each mutant (*l(3)*a. . .i) was crossed to the *J1* mutant. Female *trans*-heterozygotes (*l(3)*a. . .i/*J1*) were collected and mated to *DfB81Dp124P/TM3* males. Each mutant allele is lethal in combination with *DfB81Dp124P*; therefore, if the *l(3)*a. . .i maps to the region uncovered by the deficiency, the frequency of recombination between the two mutations would be very low and no recombinants (*e.g.*, *l(3)a*$^+$ *J1*$^+$*/DfB81Dp124P*) would be recovered (all progeny would have a Stubble phenotype). However, if the mutation mapped outside of the region, the frequency of recombination between the *l(3)*a. . .i and *J1* would be high generating wild type recombinant chromosomes. These would be viable over the deficiency, and progeny with wild-type bristles would eclose.

genotype *Dp(3;1)R14/X; mutant/balancer* were tested for flight ability. The adult flies were collected within six hours of eclosion and aged for 48 hours. They were tested individually by dropping each adult fly into a glass graduated cylinder coated with mineral oil (BENZER 1973). Gradations on the tube ranged from 8 at the top of the cylinder to 1 at the bottom. Flies that dropped straight through the cylinder were scored as 0. Approximately 25–50 flies of a particular genotype were scored.

Hybridization *in situ* to Drosophila salivary gland polytene chromosomes: Salivary gland chromosomes were prepared as described previously (BONNER and PARDUE 1976), except salivary glands were dissected in 45% acetic acid. Hybridization probes were generated by nick translation of plasmid DNA in the presence of [^{3}H]deoxyribonucleotides (New England Nuclear or Amersham) or [^{35}S]-deoxycytidine (New England Nuclear). The labeled DNA (5×10^5–1×10^6 cpm/slide) was hybridized to salivary gland chromosomes under siliconized coverslips in 16 μl of 50% formamide, 5 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate; pH 7.0) containing 0.5 μg/μl of yeast tRNA for 20 hr at 42°. Autoradiography and Giemsa staining were done by standard procedures.

Preparation of high molecular weight Drosophila genomic DNA, electrophoresis of DNA, and preparation of labeled DNA: High molecular weight Drosophila DNA was prepared as described previously (FALKENTHAL *et al.* 1984). Genomic DNA was digested with the appropriate restriction

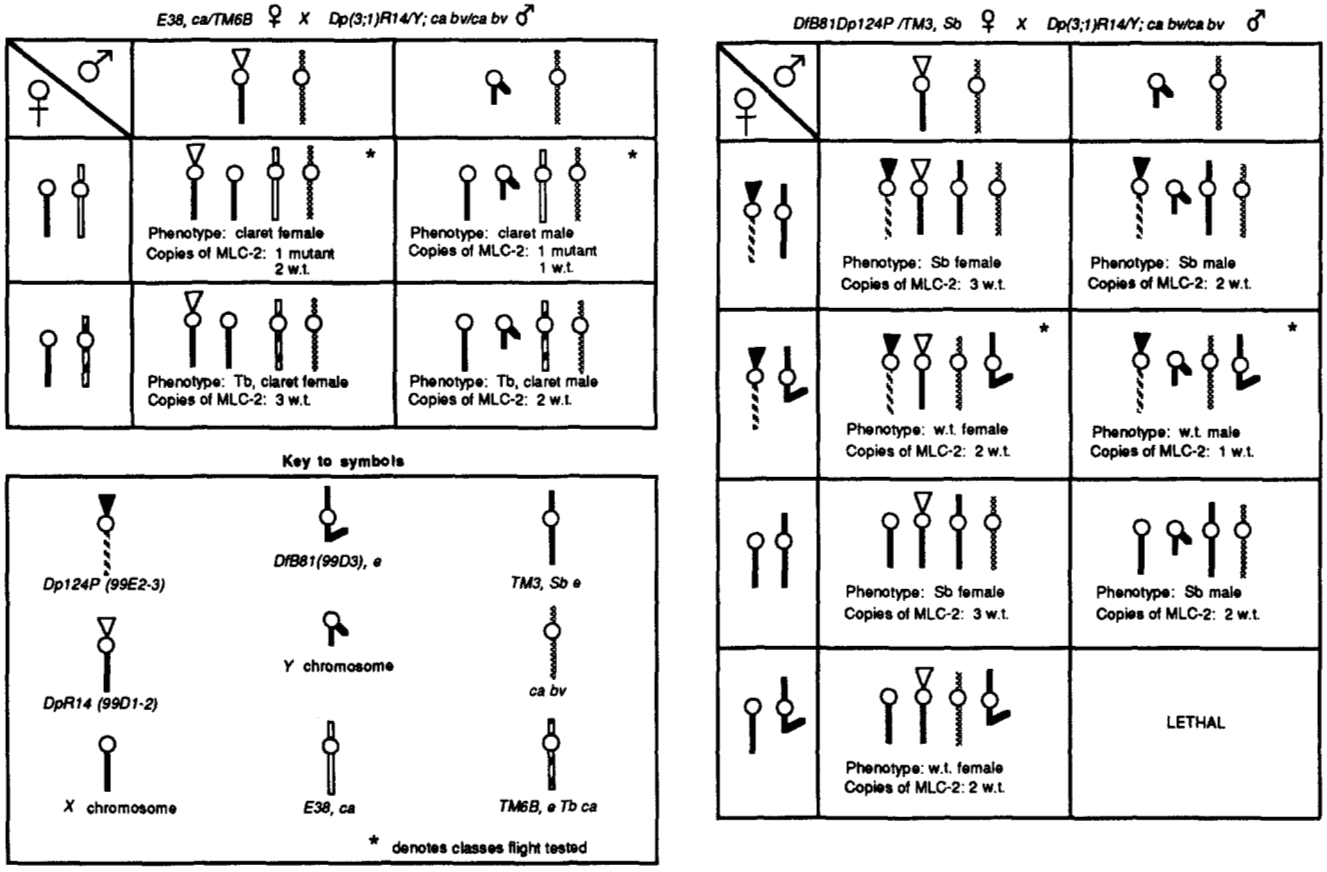

FIGURE 2.—Genetic crosses to introduce a duplication of the 99D1-2 to 100 region. *DfB81Dp124P/TM3* and *E38/TM6B* females were crossed with *Dp(3;1)R14* (99D1-2 to 100F) males to determine if the dominant flightless behavior of each could be rescued by the introduction of additional wild-type loci. As a control for genetic background, sibs of the genotype *X/Y; mutant/balancer* were tested for flight behavior to determine a baseline for phenotypic rescue.

enzymes, was displayed on 4% polyacrylamide gels (1 × TBE: 0.08 M Tris, pH 8.3, 0.08 M borate, and 0.002 M EDTA), and was transferred to a nylon membrane (Zeta-probe) by electroblotting in 0.5 × TBE at 30 V for 14–18 hr. Gel isolated DNA restriction fragments were labeled by nick-translation with α-[^{32}P]deoxycytidine (Amersham) to a specific activity of 4–10 × 10^7 dpm/μg. Prehybridization and hybridization with 10% dextran sulfate was done as described previously (MULLINS *et al.* 1978).

Isolation and electrophoresis of RNA: Total cellular RNA was isolated as described previously (CHUN and FALKENTHAL 1988). The RNA was displayed on denaturing 8 M urea-4% polyacrylamide gels. After electrophoresis the RNA was transferred to a nylon membrane (Zeta-probe) by electroblotting in 0.5 × TBE at 30 V for 14–18 hr.

A full length MLC-2 cDNA clone (pcmlc-2,13.1) was used to generate DNA hybridization probes by nick translation. The full length insert of this clone (an 810 bp *Eco*RI fragment) was cloned into pBS M13$^+$ (data not shown). Anti-sense RNA was synthesized from this subclone (pbscmlc-2) by transcription using T7 polymerase in the presence of [^{32}P]GTP. Anti-sense RNA homologous to myosin alkali light chain mRNA was synthesized using pcALCbs.1 DNA template (pBS M13+ vector containing the 880-bp *Eco*RI fragment of the full length myosin alkali light chain cDNA clone pcMLC-1.10 (FALKENTHAL *et al.* 1984)).

To quantitate the level of MLC-2 and MLC-ALK RNA the autoradiograms of the northern gels were scanned by a microbeam laser densitometer. The area under the peaks was determined for MLC-2 and MLC-ALK; and, the value for the MLC-2 was divided by that obtained for the MLC-ALK to normalize for the concentration of RNA loaded onto the polyacrylamide gel.

Electron microscopy: Dissection, fixation, and sectioning of IFM was done as described previously (CHUN and FALKENTHAL 1988). Gold sections were collected and stained with 2% uranyl acetate and Reynold's lead citrate before viewing.

RESULTS

Identification of chromosome breakpoints surrounding the myosin light chain-2 gene: The IFM is very sensitive to gene dosage. Hemizygosity of the MHC gene and the *Act88F* gene (which encodes the IFM specific actin isoform) both result in dominant flightless behavior (MOGAMI *et al.* 1986; HIROMI and HOTTA 1985). Likewise a mutation which blocks expression of the IFM specific tropomyosin isoform also exhibits dominant flightless behavior (KARLIK and FYRBERG 1985). We presumed that MLC-2 mutations would likewise exhibit dominant flightless behavior. Therefore, we mutagenized Canton-S males and screened approximately 25,000 F_1 progeny for dominant flightless behavior. We recovered twelve dominant flightless mutants mapping to seven distinct loci; however, none of these mutations mapped to the

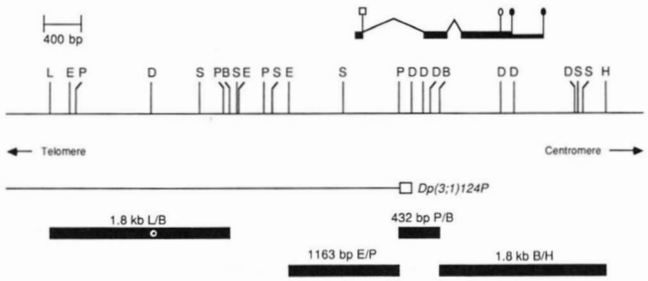

FIGURE 3.—The myosin light chain-2 locus. Restriction endonuclease map of the MLC-2 locus and flanking sequences is shown. Abbreviations for restriction endonucleases are: B, *Bam*HI; D, *Dde*I; E, *Eco*RI; H, *Hin*dIII; L, *Bcl*I; P, *Pst*I; S, *Ssp*I. Note that this map contains a correction to that previously reported by PARKER, FALKENTHAL and DAVIDSON (1985) and TOFFENETTI, MISCHKE and PARDUE (1987) which omitted the 550 bp *Eco*RI fragment upstream of the MLC-2 transcription start site. The gene encodes two mRNAs of 1100 and 1400 bp which differ only in the site of polyadenylation. Symbols used are □, translation initiation codon; ○, translation termination codon; ●, polyadenylation site. The breakpoint of *Dp(3;1)124P* maps to the 130 bp *Pst*I/*Dde*I fragment indicated by the open box. Restriction fragments used as hybridization probes are indicated below the map.

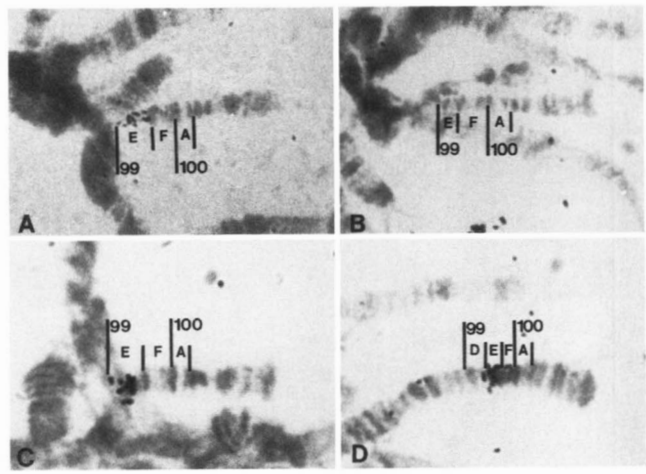

FIGURE 4.—Characterization of the *Dp(3;1)67A* and *Dp(3;1)124P* breakpoints. Hybridization *in situ* was used to localize the chromosomal breakpoints of *Dp(3;1)67A* and *Dp(3;1)124P* relative to the MLC-2 gene. The MLC-2 gene is located at 99E1-3. (A) Salivary gland chromosomes from *Dp(3;1)67A* are hybridized with [^{3}H]-labeled dmpt57 DNA, which contains the entire MLC-2 gene. Silver grains are seen at 99E near the junction of the duplication with the *X* heterochromatin. (B) Salivary gland chromosomes from *Dp(3;1)124P* are hybridized with the 1.8-kb *Bam*HI/*Hin*dIII restriction fragment which contains the 3′ end of the MLC-2 transcription unit. While no hybridization is detected (the absence of silver grains) on the duplicated arm, hybridization is detected on distal *3R* of the same chromosome spread (D). (C) Salivary gland chromosomes of *Dp(3;1)124P* are hybridized with the 1.8-kb *Bcl*I/*Bam*HI restriction fragment which contains sequences upstream of the MLC-2 gene. Hybridization is detected at the junction of the duplication with the *X* chromosome at the chromocenter.

MLC-2 locus at 99E1-3 (data not shown).

Given our inability to obtain MLC-2 mutations by F_1 screens for dominant flightless behavior, we adopted an alternative strategy of screening for recessive lethal mutations over a deficiency chromosome lacking the MLC-2 locus. KONGSUWAN, DELLAVALLE and MERRIAM (1986) have constructed a series of synthetic segmental deficiencies for the 98EF-100F region of chromosome *3* utilizing a series of *Dp(3;1)* chromosomes (FRISARDI and MACINTYRE 1984), which contain terminal portions of chromosome *3R* translocated to the short arm of the *X* chromosome, in conjunction with deficiency segregants of *T(Y;3)* chromosomes (LINDSLEY *et al.* 1972). The breakpoints of two of the duplication bearing chromosomes, *Dp(3;1)67A* and *Dp(3;1)124P*, were reported to be near the MLC-2 gene at 99E1-3. Using hybridization *in situ* with various radioactively labeled restriction fragments containing DNA upstream from and including the transcribed region of the MLC-2 gene (see Figure 3 for a description of the hybridization probes used) we found that *Dp(3;1)67A* contains the MLC-2 gene, whereas the *Dp(3;1)124P* chromosome breaks either upstream of or within the 5′ end of the MLC-2 transcription unit (Figure 4).

To determine the breakpoint of *Dp(3;1)124P* more precisely genomic DNA was isolated and analyzed by whole genome Southern analysis using three adjacent restriction fragments, which span the transcribed region of the MLC-2 gene, as hybridization probes. As indicated by the hybridization data shown in Figure 4, the breakpoint on the *Dp(3;1)124P* chromosome may have joined euchromatin from the third chromosome to heterochromatin of the *X* chromosome. Centromeric heterochromatin is underreplicated in polyploid adult tissues (ENDOW and GALL 1975); therefore, the junction fragment should be underrepresented relative to sequences on the non-rearranged third chromosome. An additional band of 5700 bp in the *Ssp*I digest and 6400 bp in the *Dde*I digest is detected in the *Dp(3;1)124P* lane as compared to the control *ca bv* lane with only two of the three hybridization probes (Figure 5). As expected for the junction fragment, the intensity of the 5700-bp band in the *Ssp*I digest and the 6400-bp band in the *Dde*I lane is low relative to the intensity of the other bands showing hybridization. Because the 1.8-kb *Bam*HI/*Hin*dIII fragment fails to hybridize to the junction fragment, we infer that the breakpoint lies upstream of the *Bam*HI site. The breakpoint must lie downstream of the *Pst*I site and upstream of the *Dde*I site within the 432-bp *Pst*I/*Bam*HI fragment based on the hybridization of the 1163-bp *Eco*RI/*Pst*I and the 432-bp *Pst*I/*Bam*HI fragments to the unique junction fragment in the *Dde*I digest (Figure 5, A and B, lane 4). From these results we conclude that the *Dp(3;1)124P* breakpoint lies within the 130-bp *Pst*I/*Dde*I fragment within intron 1 of the MLC-2 gene (Figure 3). Therefore, the *Dp(3;1)124P* chromosome contains the transcription start site of the MLC-2 gene but lacks all of the MLC-2 protein coding sequences.

Characterization of a synthetic deficiency that lacks the MLC-2 gene: Having established that *Dp(3;1)124P* does not contain MLC-2 coding se-

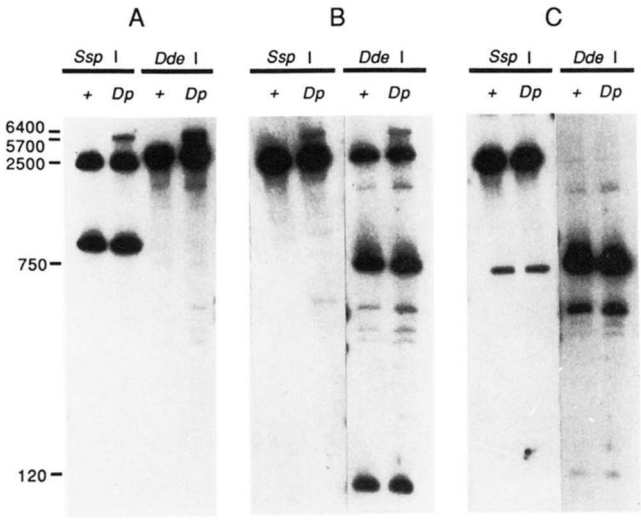

FIGURE 5.—Southern analysis of genomic DNA isolated from *Dp(3;1)124P*. Genomic DNA was isolated from *ca bv* and *Dp(3;1)124P* adult flies. Only males were used for the isolation of DNA in the *Dp(3;1)124P* stock, because females carry an attached *X* chromosome. Because the third chromosomes in the *Dp(3;1)124P* stock are marked with *ca bv*, genomic DNA was isolated from a *ca bv* stock to use as a control. DNA from the *Dp(3;1)124P* and *ca bv* stock was digested with two enzymes, *Ssp*I and *Dde*I, that have recognition sequences expected to be contained in satellite DNA. (A) Hybridization with the 1163-bp *Eco*RI/*Pst*I restriction fragment detects one additional band in the *Dp(3;1)124P* lanes as compared to the control *ca bv* lanes. (B) Hybridization with the 432-bp *Pst*I/*Bam*HI restriction fragment detects the same additional bands in the *Dp(3;1)124P* lanes as compared to the control *ca bv* lanes. The intensity of the additional band detected with the 432-bp *Pst*I/*Bam*HI fragment was five- to tenfold less than seen with the 1163-bp *Eco*RI/*Pst*I fragment. (C) Hybridization with the 1.8-kb *Bam*HI/*Hin*dIII restriction fragment detects no additional bands in the *Dp(3;1)124P* lanes as compared to the control *ca bv* lanes.

quences, we chose the synthetic deficiency *DfB81Dp124P* for use in our genetic analysis. The *DfB81* segregant lacks material from 99D3 to the telomere and in combination with *Dp(3;1)124P* generates a synthetic deficiency of the 99D3 to 99E2-3 interval. To confirm that the expression of the MLC-2 gene is reduced in the synthetic deficiency *DfB81Dp124P/TM3* we analyzed the accumulation of MLC-2 RNA by quantitative northern gel analysis. As seen in Figure 6A, the concentration of MLC-2 RNA (normalized to that of the MLC-ALK RNA) in the *DfB81Dp124P/TM3* stock was 40.8 ± 2.0% of the concentration of the MLC-2 RNA in the *DfB81Dp67A/TM3* stock. Control experiments using RNA isolated from *Dp(3;1)124P* and a *ca bv* stock showed that, as expected, the concentration of MLC-2 mRNA in the *Dp(3;1)124P* stock was 94.4 ± 4.1% of that in the *ca bv* stock (data not shown). In addition, analysis of the accumulation of myofibrillar proteins in the IFM of *DfB81Dp124P/TM3* by two dimensional polyacrylamide gel electrophoresis showed that the accumulation of MLC-2 protein relative to that of other IFM proteins was reduced (data not shown).

Dominant flightless behavior of *DfB81Dp124P*:

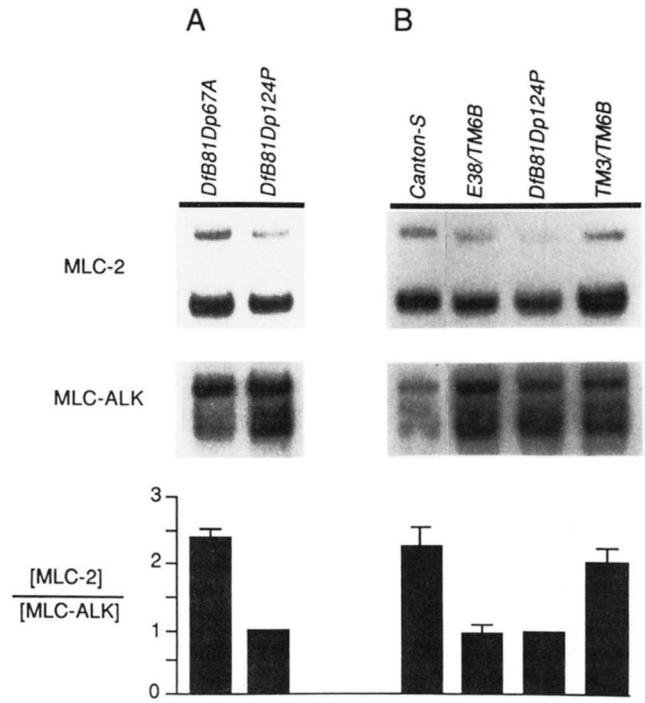

FIGURE 6.—Quantitation of MLC-2 RNA levels in the thoracic musculature of adults. (A) *DfB81Dp124P/TM3* and *DfB81Dp67A/TM3*. As a control for full diploid MLC-2 expression we analyzed the accumulation of the MLC-2 RNA in *DfB81Dp67A/TM3*, because the genetic background of this stock is identical to that of the *DfB81Dp124P/TM3* except for the location of the *Dp(3;1)* breakpoint. (B) *E38-13/TM6B*, *DfB81Dp124P/TM3*, Canton-S, and *TM3,Sb/TM6B,Tb*. Total cellular RNA was isolated from dissected thoraces of each stock. Approximately 4 μg of total RNA of each sample was separated on a 4% denaturing polyacrylamide gel and then electrophoretically transferred to a nylon membrane. MLC-2 sequences were detected on the same membrane by hybridization with [^{32}P]-labeled DNA homologous to the full length MLC-2 cDNA (see MATERIALS AND METHODS). Following autoradiography, the hybridized nucleic acid was removed from the membrane by heat deaturation. MLC-ALK sequences were detected by hybridization with [^{32}P]-labeled anti-sense MLC-ALK RNA. The relative concentration of MLC-2 in each genetic stock (normalized to the concentration of MLC-ALK mRNA) is displayed in the histogram below the autoradiograms.

The function of the IFM is very sensitive to the dosage of genes encoding structural components of the myofibril. Thus, a deficiency lacking MLC-2 coding sequences might also be expected to exhibit dominant flightless behavior. *DfB81Dp124P* (99D3 to 99E2-3) exhibits dominant flightless behavior (Figure 7). To ensure that the flightless behavior is due to hemizygosity of this interval and is not due to an induced mutation elsewhere on the chromosome, a duplication including this region, *Dp(3;1)R14*, was crossed to *DfB81Dp124P/TM3*. (See MATERIALS AND METHODS for a description of the crosses and expected progeny classes as outlined in Figure 2.) As depicted in Figure 7, the introduction of the duplication completely rescues the flightless behavior of the deficiency stock. In addition, *DfB81Dp67A* (99D3 to 99D9-E1) has normal flight behavior. Therefore, both the MLC-2 gene and dominant flightless behavior map between the break-

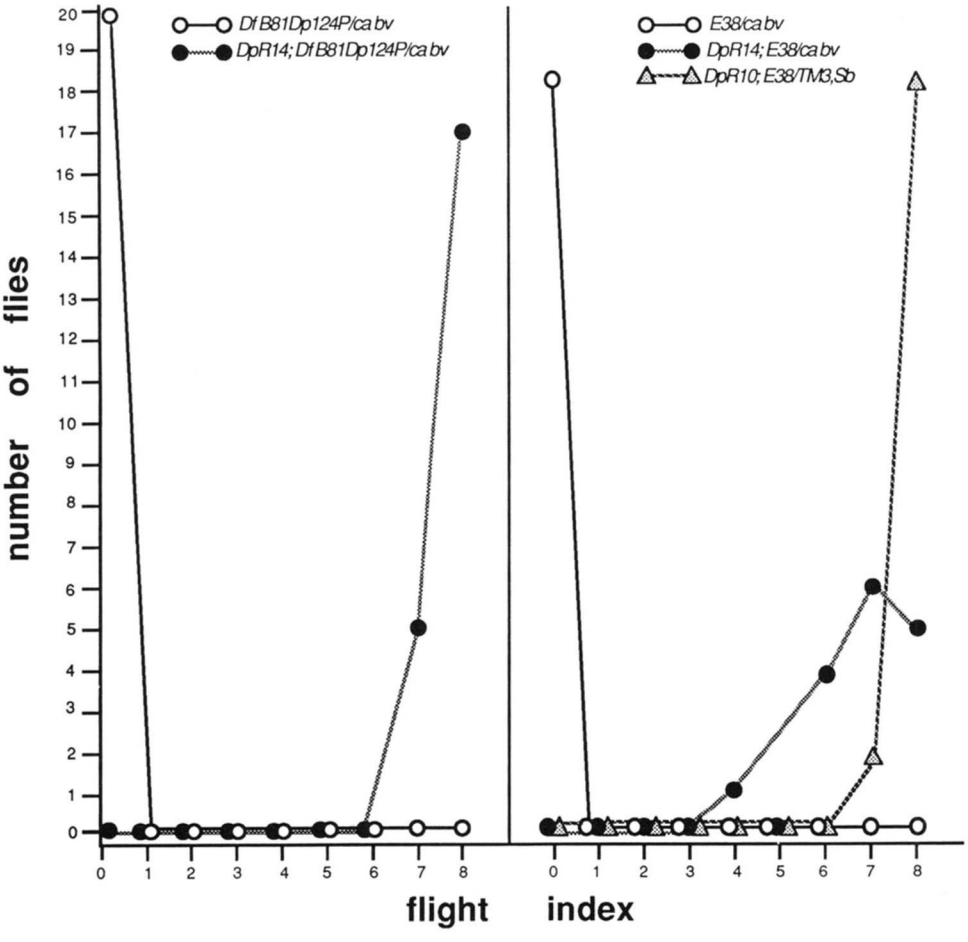

FIGURE 7.—A duplication containing *Ifm(3)99Eb*$^{+}$ rescues the dominant flightless behavior of *DfB81Dp124P* and *E38*. Progeny of the genotype *Dp(3;1)R14; DfB81Dp124/ca bv*, *Dp(3;1)R14; E38/ca bv*, and *Dp(3;1)R10;E38/ca bv* (see crosses in Figure 2) were collected 48 hr posteclosion and tested for flight (see MATERIALS AND METHODS). A flight index of 0 indicates that the adults are incapable of flying or gliding, whereas a flight index greater than 7 indicates that the adults are fully competent for flight. Intermediate flight indices indicate that the individuals have impaired flight ability; they may not be able to gain altitude, but they can glide. *DfB81Dp124P* and *E38* have flight indices of 0.0. *Dp(3;1)R14;DfB81Dp124/cabv*, *Dp(3;1)R14;E38/ca bv*, and *Dp(3;1)R10;E38/ca bv* have flight indices of 7.8 ± 0.4, 7.1 ± 1.0, and 7.9 ± 0.3, respectively.

points of *Dp(3;1)67A* and *Dp(3;1)124P* (99D9-E1 to 99E2-3).

To characterize the basis for this dominant flightless behavior, the ultrastructure of IFM myofibrils was examined by electron microscopy (Figure 8). The sarcomeric structure of *DfB81Dp124P/TM3* flies is clearly disrupted. Although sarcomere length is approximately the same as that of wild type (2.5 μm), the myofibrils appear swollen due to altered spatial arrangement of thick and thin filaments; filaments peel away from the periphery of the fibril. This is apparent in cross section where thick filaments with surrounding thin filaments are found scattered instead of in tight hexagonal packing (large arrow, Figure 8D) and where large gaps appear between bundles of filaments (small arrows, Figure 8D). While the individual thick and thin filaments do not appear affected at this level of resolution, the interaction of thick and thin filaments is clearly disrupted. Of 26 loci that are expressed at high concentration during myogenesis only the MLC-2 gene maps to this deficiency (FALKENTHAL *et al.* 1984). Therefore, it appears that a reduction in the stoichiometry of MLC-2 protein in the IFM has profound effects upon thick filament and thin filament association in the sarcomere.

In summary, the MLC-2 gene maps within a deficiency that exhibits two characteristics: dominant flightless behavior and abnormal myofibrillar structure. These observations are consistent with what would be predicted for a reduction in the amount of MLC-2. To confirm that the phenotypes of the deficiency heterozygotes are due to the reduction in MLC-2, it was necessary to isolate point mutations in the MLC-2 gene.

Recovery of recessive lethal complementation groups mapping to bands 99D3 to 99E2-3: The synthetic deficiency *DfB81Dp124P* was used to screen for EMS-induced recessive lethal mutations in the 99D3 to 99E2-3 region. Over 9300 chromosomes were tested using an F_2 screen (see MATERIALS AND METHODS for a description of the mutagenesis scheme); 44 recessive lethal mutations were recovered. To simplify the complementation analyses each mutant line was crossed to a set of nested synthetic deficiencies, *DfB81DpR10* (99D3 to 99D6-9) and *DfB81Dp67A* (99D3 to 99D9-E1), which share a common centromere proximal breakpoint (Figure 9). Altogether, the *inter se* crosses and deficiency mapping identified 23 complementation groups; many of these complementation groups contained only one mutant allele while other complementation groups had multiple alleles that showed complex complementation patterns.

To confirm the results of complementation map-

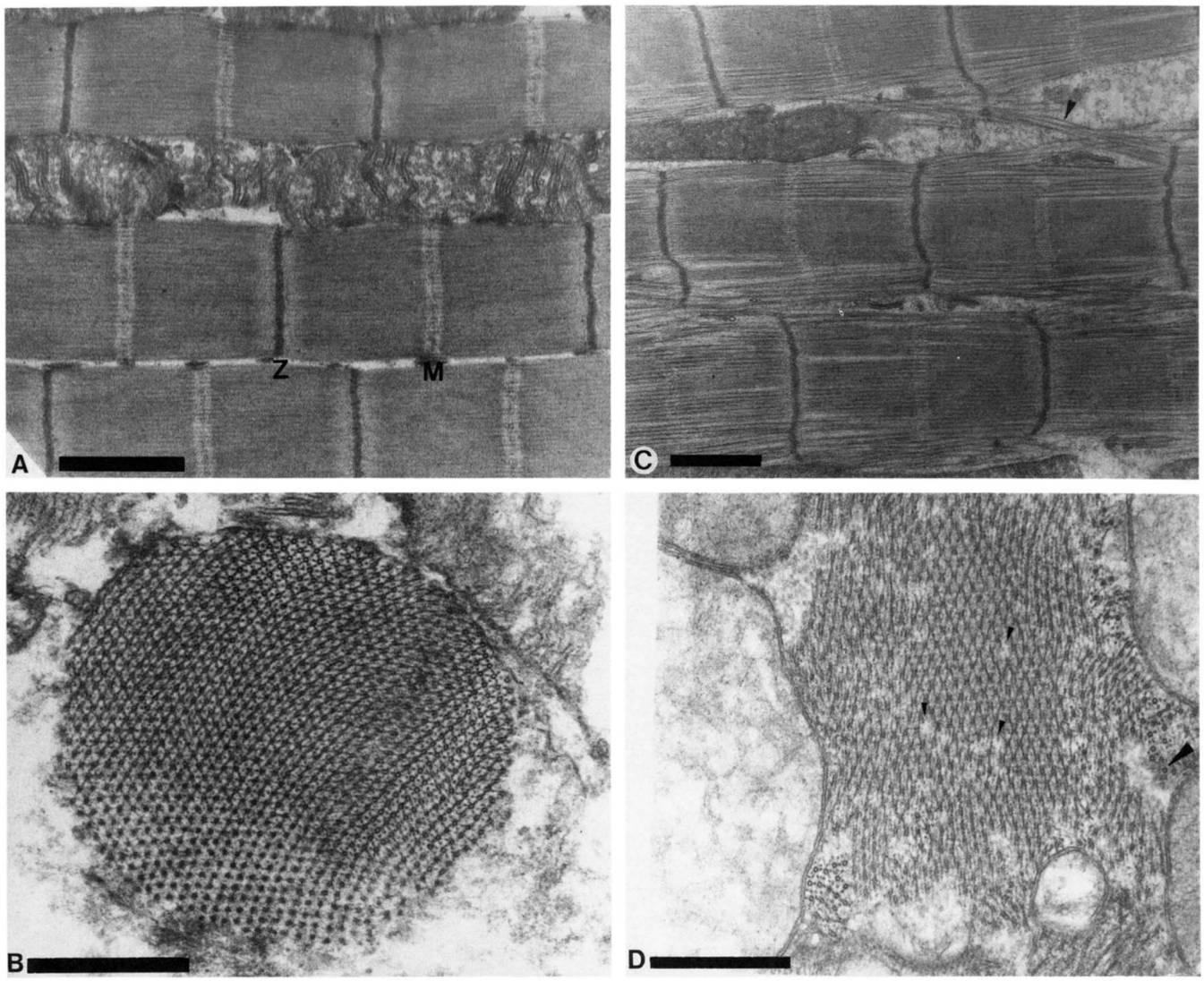

FIGURE 8.—Ultrastructure of the IFM of Canton-S and *DfB81Dp124P/TM6B* adults. (A, B) Canton-S; (C, D) *DfB81Dp124P/TM6B*. (A) Transverse section of wild type IFM shows a constant sarcomeric length of 2.7 μm. The Z discs are the most electron dense structure of the sarcomere appearing very straight and compact. The M line is well defined showing electron dense material distributed along the entire width of the sarcomere. The bar represents one micron. (B) Cross section of the myofibril shows the cylindrical aspect of the myofibril and the regular hexagonal array of thin filaments surrounding each thick filament. The bar represents 0.5 μm. (C) Transverse section of *DfB81Dp124P/TM6B* demonstrates that the sarcomere length of 2.5 μm is approximately the same as wild type. The H band (the middle region of the sarcomere where thin filaments are not found) is present; however, the associated M lines are poorly defined. If they are present, the electron dense region is dispersed. Notice that thick and thin filaments at the periphery form connections between adjacent myofibrils (arrow). Bar represents one micron. (D) Cross section reveals that the cylindrical aspect of the myofibril is lost. Thick filaments with surrounding thin filaments are found randomly positioned instead of in tight hexagonal packing (large arrow) and large gaps appear between bundles of filaments (small arrows). Bar represents 0.5 μm.

ping to 99DE, we localized single alleles of each group by recombination mapping (see MATERIALS AND METHODS). As a result of these recombination experiments all but five single member complementation groups mapped outside this region (data not shown) as did all alleles that showed complex complementation behavior. Therefore, a total of one dominant flightless and 29 recessive lethal mutations which define 12 complementation groups were identified in the 99D3 to 99E2-3 interval (Table 2). Eight complementation groups were defined genetically that map between 99D3 and 99D6-D9; two mapped between 99D6-D9 and 99D9-E1; and two mapped between 99D9-E1 and 99E2-3 (Figure 9).

Two independent mutations were induced on the *E38* chromosome: *E38* was identified by JOHN MERRIAM in an analogous and independent genetic screen using the smaller deficiency, *DfB81Dp67A*, as a tester chromosome. We noted that *E38* heterozygotes exhibit dominant flightless behavior (Figure 7) that maps centromere distal to *ca* at 102.0 map units (Figure 10). Because the deficiency that uncovers the lethal phenotype of *E38* has normal flight behavior, this suggested that the chromosome contains two distinct mutations, one recessive lethal mutation (*l(3)99Df*) mapping between 99D3 and 99D6-9 and one dominant flightless mutation mapping between 99D9-E1 and 99E2-3. We should be able to differentially rescue

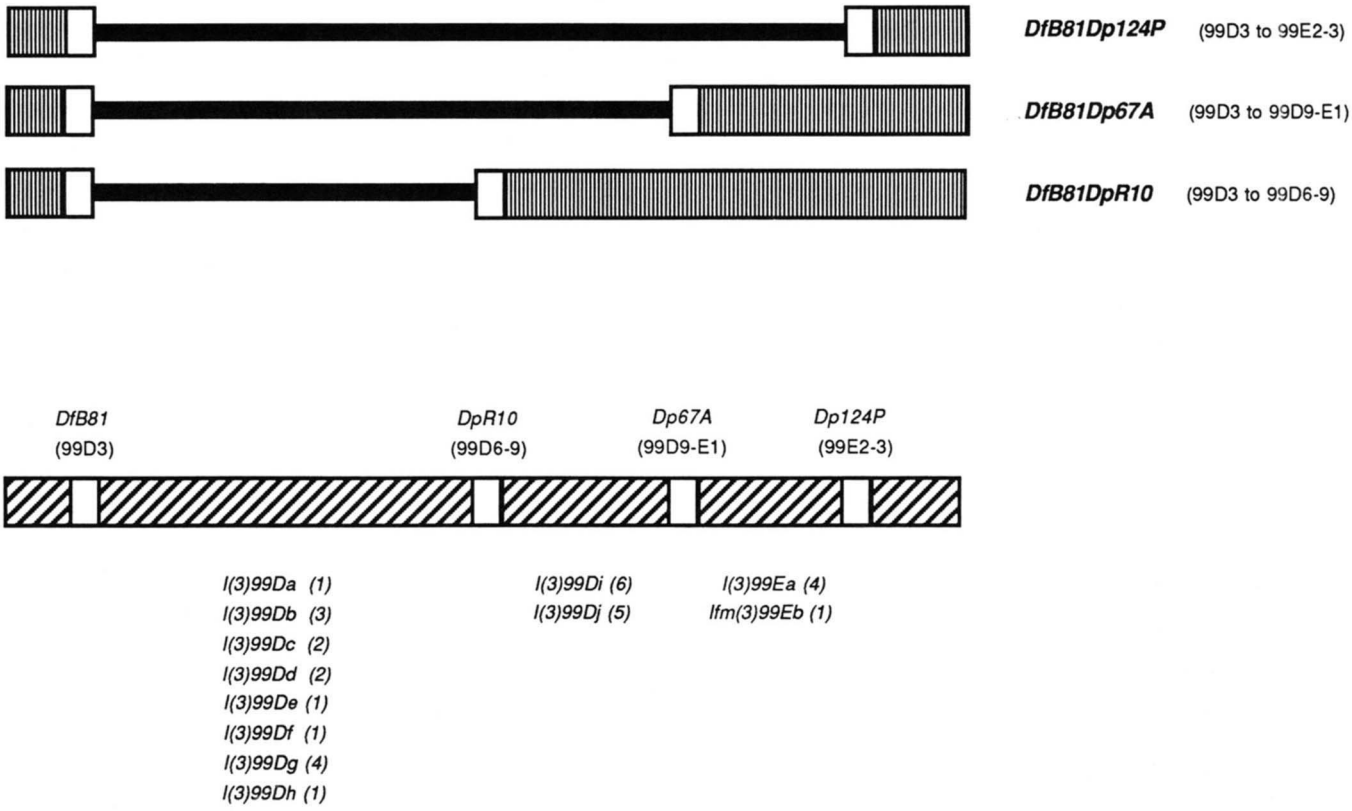

FIGURE 9.—Recessive lethal complementation groups contained within the *DfB81Dp124P* deficiency. The order and extent of a number of chromosome rearrangements in the 99D-E region as determined by cytological and genetic analysis is indicated. The cytological breakpoints are indicated in parentheses above the line representing each rearrangement. The open boxes indicate the region on the polytene chromosome within which the breakpoint lies. The complementation groups which map to each interval are indicated, as well as the number of alleles in each complementation group (numbers in parentheses).

TABLE 2

Listing of lethal complementation groups

Complementation groups	Alleles	Complementation groups	Alleles
l(3)99Da	*S5*	*l(3)99Di*	*SF4*
l(3)99Db	*SF1*		*SF7*
	SF2		*SF9*
	J2		*J5*
l(3)99Dc	*SF5*		*L4*
	L1	*l(3)99Dj*	*S1*
l(3)99Dd	*SF6*		*S3*
	J4		*J7*
l(3)99De	*J6*		*L3*
l(3)99Df	*E38*		*L7*
l(3)99Dg	*SF3*	*l(3)99Ea*	*S4*
	SF8		*J1*
	L8		*J3*
	L6		*L9*
l(3)99Dh	*L5*	*Ifm(3)99Eb*	*E38*
		l(3)nc99Eb	*J8*
			C10
			L2
			S2

the flightless behavior and recessive lethality by the introduction of either *Dp(3;1)R10* or *Dp(3;1)R14*. *DfB81DpR10* or *Dp(3;1)R14* males were crossed with *E38-13/TM6B* females. *Dp(3;1)R10;E38-13/TM3* and *Dp(3;1)R14;E38-13/ca bv* F1 females were collected, were tested for flight, and subsequently crossed to *E38-13/TM6B* or *ca,E38/TM6B* males, respectively, to test for the rescue of the recessive lethality. The introduction of both *Dp(3;1)R14* and *Dp(3;1)R10* completely rescues the flightless behavior of *E38* (Figure 7); however, only *Dp(3;1)R14* rescues the recessive lethality of *E38* (data not shown). These results indicate that the flightless behavior maps distal to the *Dp(3;1)R10* breakpoint. Because the haplo-insufficient flight behavior of *DfB81Dp124P* maps between 99D9-E1 and 99E2-3, we conclude that the second mutation on the *E38* chromosome responsible for this behavior maps to this interval. We were unable to ascertain whether the mutation responsible for the dominant flightless behavior has a recessive lethal phenotype due to the lack of a deficiency that uncovers only the 99D9-E1 to 99E2-3 region. We believe that the two mutations resulted from independent mutational events and are not the result of a single mutation, *i.e.*, an inversion, because we do not detect any rearrangements at either the cytological level or the molecular level as revealed by whole genome Southern analysis of *E38* heterozygotes using MLC-2 hybridization probes (data not shown).

Characterization of the flightless complementa-

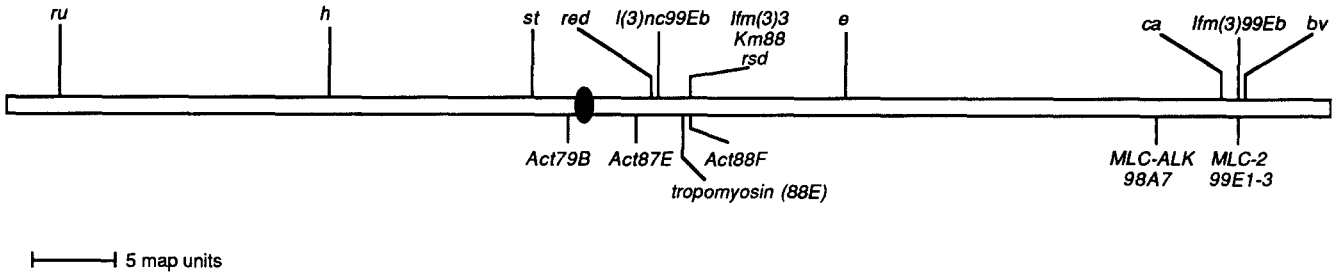

FIGURE 10.—Genetic map of chromosome *3* of *Drosophila melanogaster*. The meiotic map position for each locus is indicated above the map; the corresponding cytological locus is indicated below the map. The positions of genes encoding muscle proteins which have been cloned are indicated. The newly determined map position for the *Ifm(3)99Eb* gene (3–102.0) is indicated, as well as the position for the noncomplementing locus, *l(3)nc99Eb* (3–54.2).

TABLE 3

Complementation behavior of dominant flightless alleles[a]

Allele	Temperature (°C)	*J8*	*L2*	*S2*	*C10*	*E38*
DfB81Dp124P	18	0.5	10.4	0.5	0.6	0.4
	22	2.1	4.2	0.4	1.9	0.5
	29	5.0	6.2	<0.8[b]	16.3	ND[c]
J8	18	—	<0.2	<0.3	<0.4	25.0
	22		<0.5	<0.5	<0.7	73.2
	29		<0.5	<0.3	<0.7	78.7
L2	18		—	<0.2	<0.4	70.5
	22			<0.4	<0.6	100.0
	29			<0.4	<0.7	100.0
S2	18			—	<0.3	8.0
	22				<0.6	9.4
	29				<0.8	10.4
C10	18				—	16.3
	22					61.0
	29					100.0

[a] The percentage of expected *trans*-heterozygotes recovered from standard complementation tests are reported. Over 500 progeny were screened from each cross to determine the level of viability.

[b] Where no *trans*-heterozygotes were recovered, the viability of the *trans*-heterozygote must be less than one *trans*-heterozygote recovered divided by the expected number of *trans*-heterozygotes.

[c] Even though no *E38/DfB81Dp124P trans*-heterozygotes were recovered, because of low fecundity at 29°, we were unable to obtain a sufficient number of progeny to determine a meaningful value.

tion group *Ifm(3)99Eb*: Our initial analysis of the flight behavior of the mutants which were located in the 99D9-E1 to 99E2-3 region by deficiency mapping identified five mutations which exhibit dominant flightless behavior (*E38*, *J8*, *L2*, *S2* and *C10*). Standard complementation analysis between these mutants indicated that they define a single complementation group. As seen in Table 3, *J8*, *L2*, *S2* and *C10* showed low viability over the *DfB81Dp124P* deficiency (<11% at all temperatures tested), while no viable *trans*-heterozygotes between any two of these mutations were recovered. In contrast, *E38* exhibited the lowest viability over the *DfB81Dp124P* deficiency (<0.5%) and exhibited allele-specific complementation with the other flightless mutant alleles ranging from complete complementation to less than 10% viability.

To our surprise all alleles except *E38* showed substantial numbers of wild-type recombinants in mapping experiments with *J1* (see MATERIALS AND METHODS), indicating that these mutations must lie outside the 99D3 to 99E2-3 region. In confirmation of these results, the flightless behavior of each mutant was mapped by recombination (see MATERIALS AND METHODS for the details of the flight mapping) to 54.2 ± 2.7 map units (Figure 10) within 2 map units of an actin gene and the tropomyosin genes (see DISCUSSION). We conclude that *J8*, *L2*, *S2* and *C10* are alleles of a noncomplementing locus, *l(3)nc99Eb*, that exhibits allele specific dominant lethal synergism with *E38*.

Only two complementation groups map to the same chromosomal region, 99D9-E1 to 99E2-3, as the haplo-insufficient flight behavior and the MLC-2 gene. All of the *l(3)99Ea* alleles are wild type for flight behavior, whereas the single allele *E38* exhibits dominant flightless behavior. Therefore, we propose one complementation group is solely responsible for the haplo-insufficient flight behavior of *DfB81Dp124P/TM3* and designate this complementation group as *Ifm(3)99Eb*.

Accumulation of MLC-2 RNA in *E38* adult thoracic musculature: The rescue of the dominant flightless behavior of *E38* by the introduction of a duplication indicates that this phenotype is due to a reduction in the amount of *Ifm(3)99Eb*$^{+}$ gene product. A hypomorphic mutation could reduce gene activity by reducing the steady state concentration of the gene product by changing either the rate of synthesis, the stability of the RNA or encoded protein, or the activity of the protein. We assessed the effect of this mutation upon the accumulation of MLC-2 RNA in adult thoracic musculature. As shown in Figure 6B, the concentration of MLC-2 RNA (normalized to that of the MLC-ALK RNA) in *E38-13/TM6B* and *DfB81Dp124P/TM3* adult thoraces is equivalent, 41 ± 7% and 45 ± 6% of the concentration in Canton-S, respectively. Therefore, the *Ifm(3)99Eb*^{E38} allele re-

sults in decreased accumulation of MLC-2 mRNA in adult thoracic musculature.

DISCUSSION

Identification of a complementation group responsible for the haplo-insufficient flight behavior of *DfB81Dp124P*: The main purpose of our genetic analysis of the 99D3 to 99E2-3 region was to isolate mutations in the MLC-2 gene. By genomic Southern analysis we have shown that a deficiency for this region, *DfB81Dp124P*, lacks the protein coding sequences of the MLC-2 gene. This mapping is consistent with our observations that there is a 50% reduction in MLC-2 RNA accumulation in the adult thorax and a concommitant decrease in the accumulation of MLC-2 protein in the IFM. We presume that the disruption of sarcomere organization in the IFM of *DfB81Dp124P* is a consequence of the decrease in MLC-2 gene expression. This supposition is supported by the mapping of both the dominant flightless behavior and the MLC-2 gene to the 99D9-E1 to 99E2-3 subinterval.

To confirm our hypothesis and isolate mutations in the MLC-2 gene, the synthetic deficiency *DfB81Dp124P* was used to isolate recessive lethal mutations that map within this interval. We identified 29 recessive lethal mutations that define eleven essential complementation groups. Because mutations mapping to *M(3)99D*, the locus encoding ribosomal protein 49, would not be recovered in our screen, there are at least 12 essential loci within this eight- to nine-band region. Analysis of the resulting mutations confirmed that only one (*Ifm(3)99Eb*) of two complementation groups mapping between 99D9-E1 and 99E2-3 exhibits dominant flightless behavior. Quantitative northern gel analysis demonstrated that the concentration of MLC-2 RNA is decreased approximately 45% relative to MLC-ALK RNA in the thoracic musculature of adults heterozygous for the hypomorphic $Ifm(3)99Eb^{E38}$ allele. On the basis of these results we propose that *Ifm(3)99Eb* encodes MLC-2.

Sarcomere assembly in flies deficient for MLC-2 protein: Thick filament assembly is independent of thin filament assembly; however, the assembly of both filament systems is required for the proper alignment and registry of Z bands (MAHAFFEY *et al.* 1985; CHUN and FALKENTHAL 1988; O'DONNELL and BERNSTEIN 1988). We presume that the interaction of thick and thin filaments via the myosin crossbridge, which consists of the globular head of the myosin heavy chain and associated MLC-2 and MLC-ALK proteins, is responsible for the correct registration of the sarcomeres. Surprisingly, the absence of tropomyosin during assembly does not grossly affect the structure of sarcomeres or the accumulation of other myofibrillar proteins, but does effect the ability of the fibrils to withstand stress (KARLIK and FYRBERG 1985). Based on the function of tropomyosin, *i.e.*, to negatively regulate acto-myosin interaction, and the structure of IFM myofibrils that lack tropomyosin, we assume that in the absence of tropomyosin thin and thick filaments associate normally during assembly.

We presume that a decrease in the amount of MLC-2 protein in the *DfB81Dp124P* heterozygotes results in myosin heads (crossbridges) lacking MLC-2 protein. Because MLC-2 is implicated in regulating myosin Mg^{2+}ATPase activity as well as maintaining the ordered helical array of myosin heads within the thick filament (SELLERS and ADELSTEIN 1987; VIBERT and CRAIG 1985), the decrease in MLC-2 protein concentration could alter the interaction of thick and thin filaments by altering the activity of the crossbridge. In fact isolated single dorsal longitudinal muscles from *DfB81Dp124P* heterozygotes contract very slowly as compared to wild type indicating that many crossbridges are not cycling (D. MAUGHAN, personal communication). These results imply that correct MLC-2/MHC stoichiometry is critical for proper functioning of the myosin crossbridge and that the myosin crossbridge plays a critical role in sarcomere assembly.

A complete understanding of the effect of decreased expression of MLC-2 upon sarcomere structure necessitates determining the concentration of MLC-2 protein within the sarcomere of *DfB81Dp124P* heterozygotes, *i.e.*, do the thick filaments which splay at the periphery of the fibril contain less MLC-2 protein than the thick filaments at the center of the fibril? We will address this issue in future studies using the emerging technology of ultrathin frozen sectioning combined with immunogold detection techniques.

Identification of a locus exhibiting dominant lethal synergism with *Ifm(3)99Eb*: Analysis of the mutations recovered over *DfB81Dp124P* identified four alleles of a second-site noncomplementing locus, which we have designated *l(3)nc99Eb*. These alleles exhibit dominant flightless behavior and show allele specific complementation with $Ifm(3)99Eb^{E38}$ and *DfB81Dp124P*. This complementation group maps distal to *red* at 54.2 ± 2.7 map units.

All of the third chromosome dominant flightless mutants that were isolated by MOGAMI and HOTTA (1981) are homozygous viable and map to the 88E-F region. Four of these mutants map to *Act88F*, the IFM specific actin gene (KARLIK, COUTU and FYRBERG 1985; HIROMI and HOTTA 1985; KARLIK, SAVILLE and FYRBERG 1987), whereas two map to the tropomyosin gene (KARLIK and FYRBERG 1985). By meiotic mapping *Act88F* is located at 57.0–57.1 map units (HIROMI and HOTTA 1985; MAHAFFEY *et al.* 1985), whereas tropomyosin is located approximately 140-kb centromere proximal to *Act88F* (KARLIK *et al.* 1984). The assumption that 1 map unit is approximately 250 kb (KIDD, LOCKETT and YOUNG 1983) places the tropomyosin genes at 56.4 map units.

Noncomplementing mutations that map to distinct genetic loci have been proposed to identify genes that encode products that participate in the same function or that interact in the same macromolecular complex (RAFF and FULLER 1984; REGAN and FULLER 1988; STEARNS and BOTSTEIN 1988). Genetic analysis of *haywire*, a noncomplementing locus of β_2-*tubulin* (RAFF and FULLER 1984; REGAN and FULLER 1988) demonstrated that the failure of second-site loci to complement β_2-*tubulin* mutations is allele specific. The general rules that came from this analysis are: (1) noncomplementation results when one allele is a hypomorph or amorph, whereas the second site allele is a neomorph or antimorph; (2) noncomplementation can occur when both alleles are antimorphs; and (3) when both alleles are amorphs, they exhibit full complementation.

As stated above, myosin interacts with actin via the myosin crossbridge. The troponin complex assembles with tropomyosin exhibiting a 39-nm periodicity along the thin filaments. Rigor crossbridges in invertebrate muscle are found with the same periodicity (TAYLOR *et al.* 1984). Mutations in the genes encoding these proteins might show lethal interactions. In fact, mutations mapping to the myosin heavy chain gene (*Ifm(2)3, Bsh*, and *Nup*) show lethal interactions with a mutation mapping to the troponin I gene (hdp^2) (HOMYK and EMERSON 1988; C. J. BELL and E. A. FYRBERG, personal communication). Both the *Act88F* and tropomyosin genes are closely linked to *l(3)nc99Eb*. Because *Act88F* is dispensible for viability, *l(3)nc99Eb* cannot be an actin mutant. It is possible that *l(3)nc99Eb* encodes tropomyosin based on its tight linkage with the tropomyosin locus, but they may be distinct loci. *l(3)nc99Eb* could map to one of the 15–16 transcription units of unknown function which flank the actin and tropomyosin genes at 88E-F (KARLIK *et al.* 1984). We are initiating a molecular characterization of *l(3)nc99Eb* which will clarify the interaction of the *l(3)nc99Eb* protein product with MLC-2, and define the role of the *l(3)nc99Eb* protein product in the structure and function of the sarcomere.

Summary: From a genetic analysis of the 99D3 to 99E2-3 region, we have identified eleven essential complementation groups. In addition, one of the identified complementation groups, *Ifm(3)99Eb*, when mutated exhibits dominant flightless behavior. We provisionally assign $Ifm(3)99Eb^{E38}$ as a hypomorphic allele of the MLC-2 gene for the following reasons: (1) $Ifm(3)99Eb^{E38}$ maps cytologically to the same region as the MLC-2 gene; (2) $Ifm(3)99Eb^{E38}$ defines the only dominant flightless complementation group uncovered by a deficiency lacking the MLC-2 gene; (3) the flightless behavior of $Ifm(3)99Eb^{E38}$ is rescued by a duplication carrying a wild type copy of *Ifm(3)99Eb*; (4) of 26 loci that are expressed at high concentration during myogenesis (FALKENTHAL *et al.* 1984), only the MLC-2 gene is located in the 99D3 to 99E2-3 region; and (5) MLC-2 gene expression is decreased in the thoraces of adult $Ifm(3)99Eb^{E38}$ heterozygotes. Definitive proof that $Ifm(3)99Eb^{E38}$ is a MLC-2 allele will come from experiments which show that the introduction of a wild type copy of the MLC-2 gene by germline transformation rescues the dominant flightless behavior of $Ifm(3)99Eb^{E38}$ and from experiments which identify the $Ifm(3)99Eb^{E38}$ mutation on the MLC-2 molecular map.

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