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Transformation and rescue of a flightless *Drosophila* tropomyosin mutant

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In the *Drosophila* flightless mutant *Ifm(3)3*, a transposable element inserted into the alternatively spliced fourth exon of the tropomyosin I (TmI) gene prevents proper expression of Ifm-TmI, the tropomyosin isoform found in indirect flight muscle. We have rescued the flightless phenotype of *Ifm(3)3* flies using P-element-mediated transformation with a segment of the *Drosophila* genome containing the wild-type TmI gene plus 2.5 kb of 5′ flanking and 2 kb of 3′ flanking DNA. The inserted TmI gene is expressed with the proper developmental and tissue specificity, although its level of expression varies among the five transformed lines examined. These conclusions are based on analyses of flight, myofibrillar morphology, and TmI RNA and protein levels. A minimum of two copies of the inserted TmI gene per cell is necessary to restore flight to most of the flies in each line. We also show that the Ifm-TmI isoform is expressed in the leg muscle of wild-type flies and is decreased in *Ifm(3)3* leg muscle. Homozygous *Ifm(3)3* mutants do not jump. The ability to jump can be restored with a single copy of the wild-type TmI gene per cell.

Key words: Drosophila/flightless mutant/gene expression/P-element transformation/tropomyosin gene

Introduction

Tropomyosin is one of the major contractile proteins in the thin filaments of muscle myofibrils. It also is a component of non-muscle cells. In muscle, tropomyosin acts in concert with troponin to regulate the response of actin and myosin to changes in the calcium concentration (Phillips *et al.*, 1986; see Squire, 1983 and Payne and Rudnick, 1984 for reviews). In *Drosophila*, two tropomyosin genes located within the 88F chromosomal subdivision encode multiple tropomyosin isoforms (Bautch *et al.*, 1982; Bautch and Storti, 1983; Basi *et al.*, 1984; Karlik and Fyrberg, 1986). The complete structure of the tropomyosin I (TmI) gene has been described and exhibits several interesting features (Basi and Storti, 1986). First, the TmI gene appears to have an unusual promoter. The 5′ flanking region lacks the characteristic 'TATA box' found in most eukaryotic promoters, but does contain many short direct repeats and a series of runs of the dinucleotide dTG. Second, the TmI gene encodes two developmentally regulated muscle TmI isoforms by alternately splicing the fourth of its five exons (Basi *et al.*, 1984). In the fibrillar indirect flight muscle (Ifm) of the adult thorax, the mature TmI mRNA includes exon 4 which encodes the last 27 amino acids of the Ifm-TmI isoform. In other non-fibrillar or supercontractile muscles (Scm), exon 4 is spliced out of the mature RNA, and the last 27 amino acids of the Scm-TmI isoform are encoded by the fifth exon. A third feature of the TmI gene is the presence of two clusters of poly-adenylation sites located 255 bp apart at the 3′ end of the gene (Boardman *et al.*, 1985). Investigations of TmI gene regulation at the levels of promoter function, differential splicing, and poly-adenylation will contribute to a general understanding of RNA synthesis and processing, and help elucidate the specific controls operating on the TmI gene during myogenesis.

We have begun to study the regulation of TmI gene expression using the P-element-mediated germ line transformation system developed by Rubin and Spradling (1982). Experiments with a variety of genes inserted via P-elements at diverse chromosomal sites have shown that these genes can be limited to one to two copies per cell, exhibit proper developmental and tissue-specific expression, and are inherited in a Mendelian manner (Goldberg *et al.*, 1983; Scholnick *et al.*, 1983; Richards *et al.*, 1983; Hazelrigg *et al.*, 1984; Cohen and Messelson, 1985). This method has advantages over both germ line transformation of mice and the myogenic tissue culture systems often used to study vertebrate muscle gene expression. In transgenic mice the number of copies of the inserted gene is difficult to control and in some cases the transgene is inappropriately expressed (Lacy *et al.*, 1983; Brinster *et al.*, 1983; Shani, 1985, 1986). The tissue culture systems may be blocked at different stages of developmental commitment or may lack trans-acting and trophic factors required for normal muscle gene expression (Seiler-Tuyns *et al.*, 1984; Gunning *et al.*, 1984; Minty *et al.*, 1986; Hickey *et al.*, 1986).

In this study we have introduced the wild-type TmI gene into the germ line of the semi-dominant flightless mutant *Ifm(3)3*. The indirect flight muscle of *Ifm(3)3* flies has fragile and poorly organized myofibrils, reduced levels of Ifm-TmI protein, and little if any normal Ifm-TmI RNA. The mutation is caused by the insertion of a transposable element into the 3′ non-coding region of the fourth exon of the TmI gene. No defects in the production of Scm-TmI RNA or protein have been noted in *Ifm(3)3* flies (Mogami and Hotta, 1981; Karlik and Fyrberg, 1985). Our analysis of five stable lines of *Ifm(3)3* transformants shows that the wild-type TmI gene restores indirect flight muscle structure and the ability to fly. A minimum of two copies of the TmI gene per cell is necessary to restore flight to most of the flies in each line. We also show that the Ifm-TmI isoform is expressed in the leg muscle of wild-type flies and is decreased in *Ifm(3)3* leg muscle. Homozygous *Ifm(3)3* mutants do not jump. The ability to jump can be restored with a single copy of the wild-type TmI gene per cell.

Results

Construction of the TmI–P-element transformation vector

We inserted the TmI gene into a slightly modified version of the P-element transformation vector pUChsneo of Steller and Pirrotta (1985). pUChsneo contains the bacterial neomycin resistance gene (*neo*) under the control of the *Drosophila* hsp70 promoter to provide a dominant selectable marker for transformation. With periodic heat shock, larvae transformed with pUChsneo grow on medium containing the neomycin analogue G418 at concentrations that kill all non-transformed larvae. We modified the

polylinker of pUChsneo and named the resulting plasmid pUC13hsneo. Approximately 9.5 kb of *Drosophila* genomic DNA were then inserted into pUC13hsneo to create the transformation vector p13/TmI. The 9.5 kb *Drosophila* DNA insert contains the entire TmI gene plus 2.5 kb of 5′ flanking and 2 kb of 3′ flanking DNA (see Figure 1 and Materials and methods).

Transformation of Ifm(3)3 mutants

Homozygous *Ifm(3)3* embryos were co-injected with p13/TmI and the helper plasmid phsπ as described by Spradling and Rubin (1982). Transformed G1 flies were isolated by their ability to grow on medium containing G418. Lines arising from different G1 transformants were balanced or made homozygous (see Materials and methods). The chromosomal locations of the P-element–TmI transposon P[TmI,neo], were determined by *in situ* hybridization to polytene chromosomes (Table I). These results and Southern blot analysis of restriction endonuclease-digested genomic DNA (data not shown) demonstrated that each of the transformed lines contains a single unrearranged copy of P[TmI,neo]. In this study we describe the characteristics of five independently transformed lines (summarized in Table I). P-[TmI,neo] is autosomal in four of the lines; in line 10-2 it is located on the X chromosome. All of the lines except 14-1 were made homozygous for the chromosome carrying P[TmI,neo]. In line 14-1 the P[TmI,neo] insert appears to be lethal when homozygous and is maintained in a balanced stock with the *MKRS* third chromosome.

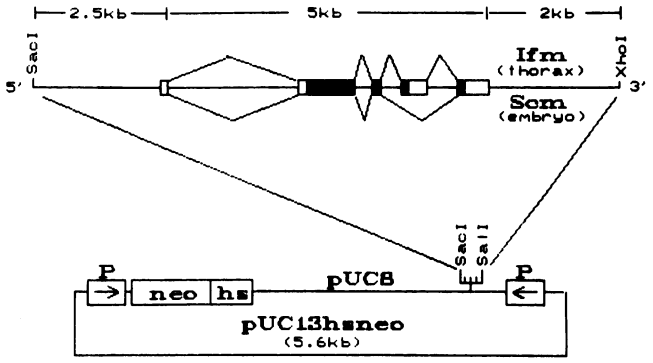

Fig. 1. The p13/TmI transformation vector. *Drosophila* genomic DNA (9.5 kb) containing the TmI gene and flanking sequences (upper part of the diagram) was inserted between the *Sac*I and *Sal*I sites of pUC13hsneo (see Materials and methods). The five exons of the TmI gene are indicated by boxes; open areas are untranslated regions, closed areas are protein coding sequence. Transcription of the TmI gene is from left to right. The exon splicing pathways which generate the mRNAs for indirect flight muscle (Ifm) and supercontractile muscle (Scm) tropomyosin isoforms are indicated. In the diagram of pUC13hsneo, the P-element termini (P), neomycin resistance gene (neo), and hsp70 promoter sequence (hs) are shown.

Phenotypic properties of the transformants

Growth on G418. Although each of the G1-transformed flies was selected by its ability to grow on medium containing G418, the lines established from different G1s do not grow equally well on the drug (Table I). Lines 11-3 and 13-2 produce numerous surviving progeny on medium containing G418, with or without heat shocking the embryos and larvae. Fewer progeny of lines 10-2 and 12-1 survive on the drug even with heat shock, and the generation time is longer. Line 14-1 grows very poorly on G418, producing only a few survivors each generation. All of the transformed lines grow as well as wild-type flies on non-selective medium. Non-uniform growth rates of different transformed lines on G418 were also observed by Steller and Pirrotta (1985) when they described transformation with a plasmid similar to pUChsneo.

Restoration of jumping and flight. Ifm(3)3 was isolated by virtue of its dominant flightless phenotype (Mogami and Hotta, 1981). Therefore, we expected to see no phenotypic difference between heterozygous transformants and *Ifm(3)3* homozygotes even if the inserted TmI gene was functional. However, we observed that transformed G1 heterozygotes and *Ifm(3)3* flies did differ in mobility. Tests of the first G418-resistant G1 flies we obtained showed that they could jump but not fly. *Ifm(3)3* flies do not jump. Subsequent examination of Canton S (wild-type)/*Ifm(3)3* heterozygotes confirmed that a single copy of the wild-type TmI gene per diploid cell confers the ability to jump but not the abili-

Table I. Characteristics of TmI transformants

Line	P[TmI,neo] location	Growth[a] on G418	Flight[b]		Jumping[c]	Relative RNA expression[d]		
			Hom.	Het. *Ifm(3)3*	Het. *Ifm(3)3*	1.7 kb (Ifm-TmI)	1.3 kb (Scm-TmI)	*neo*
Canton S		−	♀ + ♂ 56	0	56	100	n.d.	0
			♀ 46	n.d.	n.d.	100	n.d.	n.d.
			♂ 42	n.d.	n.d.	n.d.	n.d.	n.d.
Ifm(3)3		−	0			0	100	0
10-2	X(1B)	+	♀ 48	n.d.	n.d.	73	n.d.	n.d.
			♂ 36	n.d.	n.d.	n.d.	n.d.	n.d.
11-3	3R(90A)	+++	56	0	42	72	163	100
12-1	3L(71-73)	+	53	9	62	86	212	54
13-2	2R(48F-49A)	+++	52	0	0	62	143	41
14-1	3R(84D-F)	+/−	[e]	0	0	57[f]	147[f]	78

[a]+++, Numerous progeny survive on G418 without heat shock; +, fewer progeny survive on G418; +/−, poor growth on G418 even with heat shock.
[b]Percentage of flies landing on the sides of the flight tester above the swing (Figure 2). This number is an estimate of the minimum percentage of fliers in the population. As far as we can tell for example, all Canton S flies are able to fly. The numbers given in these columns are useful when comparing flying ability in populations containing both fliers and non-fliers, since only fliers can reach this region of the flight tester. Hom., homozygous; Het., heterozygous with *Ifm(3)3*.
[c]Percentage of flies landing on the walls of the flight tester below the swing when heterozygous (Het.) with *Ifm(3)3* (Figure 2).
[d]Determined from densitometric scans of a range of exposures of the RNA gel blots shown in Figures 3 and 5 (see Materials and methods).
[e]Homozygous lethal.
[f]The actual levels obtained were doubled for comparison with homozygous transformants.
n.d., Not determined.

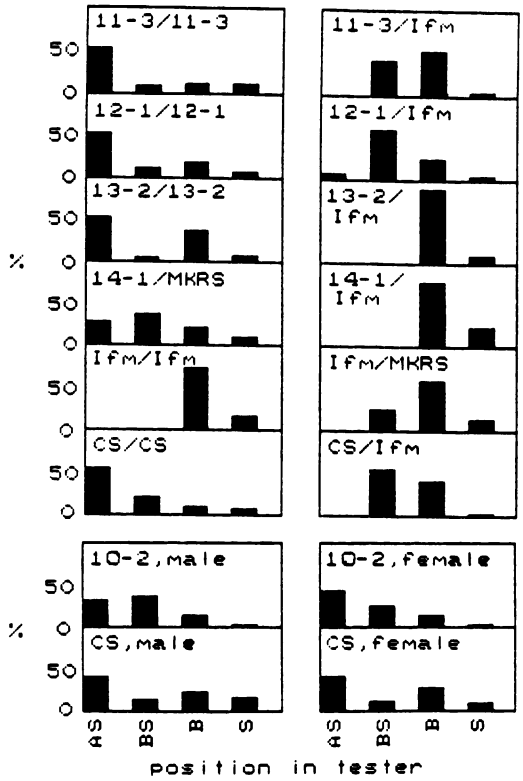

Fig. 2. Flight test results. The graphs show the percentage of flies (ordinate) landing on the sides of the flight tester above the level of the swing (AS), at or below the level of the swing (BS), and on the bottom of the tester (B). The percentage of flies remaining on the swing (S) is also shown. The genotype of the flies used in each test is indicated. 10-2, 11-3, 12-1, 13-2, and 14-1 are different lines of transformants. CS, Canton S (wild-type); Ifm, *Ifm(3)3*; MKRS, a third chromosome balancer strain containing a wild-type TmI gene. Homozygous Canton S and 10-2 flies were used in the tests of separated females and males (lower four panels).

ty to fly. These results suggested that the *Ifm(3)3* mutation affects not only indirect flight muscle but also the leg muscles involved in jumping.

When the transformed stocks were made homozygous it was clear that two copies of the P[TmI,neo] insert per cell restored the ability to fly. To examine the jumping and flying ability of the different transformed lines, we used a flight tester similar to that described by Okamoto *et al.* (1986). Figure 2 and Table I summarize these results. Our flight tester is divided into three regions. The sides of the tester above the level of the swing (AS) can only be reached by flight. The sides below the level of the swing (BS) can be reached by both jumping and flight. Flies that can jump are also found scattered over the bottom (B) of the tester, while flies that neither fly nor jump are found on the bottom directly below the swing.

Three different phenotypes can clearly be distinguished by comparing the flight tester profiles of Canton S, *Ifm(3)3* and Canton S/*Ifm(3)3* flies. Most of the wild-type flies landed on the sides of the tester, either in the region above the level of the swing (56%) or in the region below the level of the swing (22%). In contrast, all *Ifm(3)3* homozygotes ended up at the bottom of the tester directly below the swing. Canton S/*Ifm(3)3* flies can not fly but are able to jump. Thus, none of these flies reached the region above the swing. Instead 56% were found on the sides of the tester below the swing and the rest were scattered across the bottom of the tester.

The effects of one copy of the inserted TmI gene can be seen by examining the flight tester profiles of heterozygous transformants having one copy of P[TmI,neo] and two copies of the *Ifm(3)3* TmI gene per cell. Most 11-3/*Ifm(3)3* flies were found on the sides of the tester below the swing or scattered across the bottom. These results resemble those obtained with Canton S/*Ifm(3)3* flies. The majority of 12-1/*Ifm(3)3* flies also landed on the sides of the tester below the level of the swing, demonstrating that they can at least jump. However, 9% of the 12-1/*Ifm(3)3* population reached the sides of the tester above the swing. Thus, some 12-1/*Ifm(3)3* heterozygotes are able to fly. This result suggests that the inserted TmI gene is expressed at a higher level in line 12-1 than in line 11-3. 13-2/*Ifm(3)3* and 14-1/*Ifm(3)3* heterozygotes behaved exactly like *Ifm(3)3* homozygotes in the flight tester, suggesting that the inserted TmI gene in these two lines may be underexpressed relative to both the wild-type TmI gene in Canton S/*Ifm(3)3* flies and the inserted TmI genes in lines 11-3 and 12-1. Alternatively, the inserted TmI gene may be improperly regulated in the leg muscle of lines 13-2 and 14-1.

In homozygous transformants with two copies of P[TmI,neo] per cell, the phenotypic differences among lines were not as apparent. More than 50% of the wild-type flies and the flies in lines 11-3, 12-1 and 13-2 landed above the level of the swing. Underexpression of the inserted TmI gene in line 13-2 may be reflected in the relatively large number of flies which ended up scattered on the bottom of the tester.

As mentioned above, 14-1/*Ifm(3)3* flies neither jump nor fly. Because the chromosome carrying P[TmI,neo] in line 14-1 is lethal when homozygous, we determined whether the inserted TmI gene is functional in this line by comparing the behavior of the balanced stock 14-1/*MKRS* with that of *Ifm(3)3*/*MKRS* flies in the flight tester. Both of these populations contain one wild-type TmI gene from the *MKRS* chromosome and one *Ifm(3)3* TmI gene per diploid cell. 14-1/*MKRS* flies contain one copy of P[TmI,neo] per cell as well. *Ifm(3)3*/*MKRS* heterozygotes are not able to fly, but at least 30% of the 14-1/*MKRS* population can fly. These results indicate that the inserted TmI gene is active in line 14-1.

In *Drosophila*, X-linked genes in males undergo dosage compensation to produce as much product as the two active genes in females (Lucchesi, 1977; Baker and Belote, 1983). To determine if the inserted TmI gene on the X chromosome in line 10-2 undergoes dosage compensation, we compared the flight of wild-type and 10-2 males and homozygous females. Males and females were separated after anesthetizing flies with triethylamine 4 days before testing. Since some flies do not completely recover from the anesthetic, these results are not comparable with those obtained with flies that had never been anesthetized. Figure 2 and Table I show that wild-type males, wild-type females and 10-2 females have similar flight tester profiles, with 42–48% of the flies landing on the sides of the tester above the level of the swing. Since 36% of 10-2 males also landed in this region, we conclude that 10-2 males can fly, but not quite as well as 10-2 females or wild-type flies. These results suggest that partial dosage compensation of the inserted TmI gene occurs in 10-2 males.

TmI and neo RNA levels in transformed flies

Expression of the TmI and neomycin resistance (*neo*) genes in the transformants was assayed directly by RNA analysis. In these experiments total RNA was electrophoresed on formaldehyde/agarose gels, transferred to nitrocellulose filters and hybridized with plasmid DNA probes labelled with ^{32}P by nick-translation.

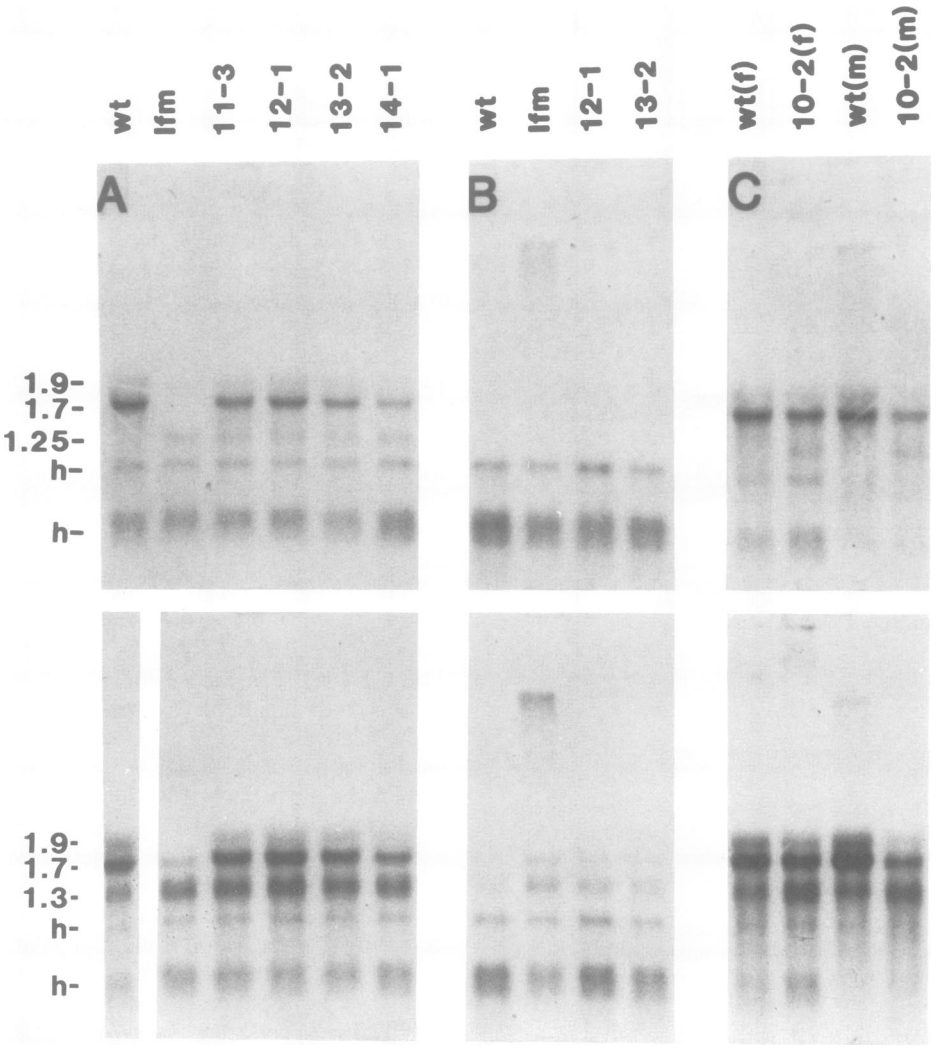

Fig. 3. Autoradiographs illustrating the TmI transcripts in total RNA from wild-type, *Ifm(3)3*, and transformed flies. Four micrograms of total fly RNA were electrophoresed on formaldehyde/agarose gels and blotted to nitrocellulose filters. The filters in the upper panels were hybridized with the exon 4 DNA probe to detect the 1.25, 1.7 and 1.9 kb Ifm-TmI transcripts. The filters in the lower panels were hybridized with plasmid DNA containing almost the entire TmI gene. This probe detects both the 1.3 and 1.6 kb Scm-TmI RNAs and the Ifm-TmI transcripts. Included in the hybridizations to both sets of blots was plasmid DNA containing the five *Drosophila* histone genes. Each probe was labelled with ^{32}P by nick translation. The RNA was obtained from **(A)** 1–3-day-old flies; **(B)** 9–12-day-old flies; **(C)** 0–3-day-old females (f) and males (m). The RNAs shown in **panels A** and **B** were prepared from populations containing equal numbers of males and females. Each of the transformed lines except 14-1 was homozygous for P[TmI,neo]. Line 14-1 contained one copy of P[TmI,neo] in the *Ifm(3)3* background. wt, Canton S; Ifm, *Ifm(3)3*; h, histone RNA. The high mol. wt material hybridizing in some of the samples in this figure is inconsistently observed in different RNA preparations.

To control for inadvertent differences in the amount of RNA loaded in different lanes, ^{32}P-labelled plasmid DNA containing the *Drosophila* histone genes was included in each hybridization. To determine relative RNA levels, a range of autoradiographic exposures of selected blots was scanned with a densitometer. With the exception of line 14-1, each stock of transformed flies used in these studies was homozygous for P[TmI,neo]. Line 14-1 contained one copy of P[TmI,neo] in the *Ifm(3)3* background.

In earlier reports we showed that the Ifm-TmI isoform (previously referred to as the thorax isoform) is encoded by mRNAs of 1.7 and 1.9 kb which comprise all five exons of the gene (Basi *et al.*, 1984; Basi and Storti, 1986). *Ifm(3)3* flies have no detectable 1.7 and 1.9 kb transcripts, but do contain low levels of aberrant 1.25 and 10 kb Ifm-TmI RNAs [see Figure 3 and Karlik and Fyrberg (1985)]. The Scm-TmI isoform (previously referred to as the embryonic isoform) is encoded by mRNAs of 1.3 and 1.6 kb which, as a result of alternative splicing, lack the fourth exon. To analyse TmI RNA, duplicate RNA blots were hybridized either with a probe containing only the fourth exon and parts of its flanking introns or with a probe containing most of the TmI gene (see Materials and methods). The exon 4 probe allowed us to distinguish the 1.25, 1.7 and 1.9 kb Ifm-TmI transcripts from the 1.3 and 1.6 kb Scm-TmI RNAs.

The top panel of Figure 3A compares Ifm-TmI expression in total RNA from 1–3-day-old wild-type, *Ifm(3)3* and transformed flies. It can be seen that the abundant 1.7 and minor 1.9 kb wild-type transcripts missing in *Ifm(3)3* RNA are restored to varying degrees in each of the transformed lines. Relative levels of the 1.7 kb mRNA obtained by densitometric scanning of autoradiographs are shown in Table I. None of the transformed lines expresses Ifm-TmI RNA at the wild-type level. Line 12-1 has the highest amount of 1.7 kb message (~86% of wild-type) and line 14-1 the least (~57% of wild-type when corrected for P-[TmI,neo] copy number). Visual inspection of Figure 3A shows

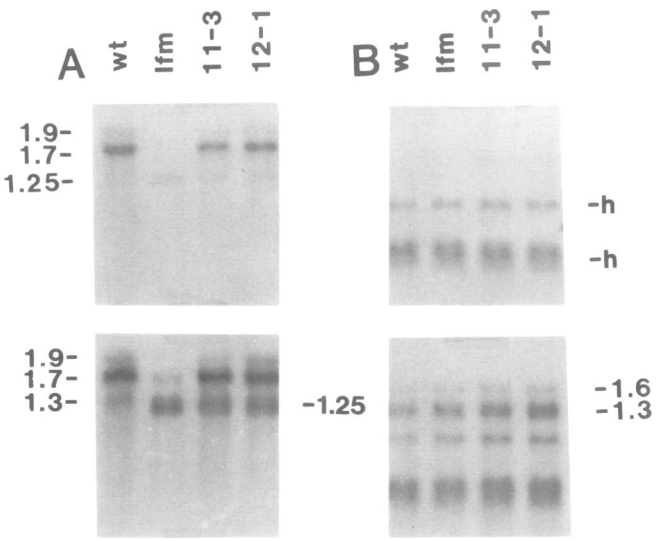

Fig. 4. Autoradiographs illustrating TmI transcripts in total RNA from thoraces and abdomens. Flies (2−6 days old) from Canton S (wt), *Ifm(3)3* (Ifm), and transformed 11-3 and 12-1 stocks were separated into thoraces **(A)** and abdomens **(B)**. Two micrograms of thoracic RNA or 4 μg of abdominal RNA were loaded on the gels. Blots in the upper panels were hybridized with the exon 4 probe. Blots in the lower panels were hybridized with the whole TmI gene probe. Hybridizations to both sets of blots contained a probe for histone RNA (h). See the legend to Figure 3 for details.

that all of the transformants have approximately the same amount of the 1.25 kb transcript as the non-transformed *Ifm(3)3* mutant. We estimate the level of the 1.25 kb transcript in the *Ifm(3)3* RNA shown in Figure 3A to be 46% of the level of the 1.7 kb transcript in wild-type RNA. This number has been corrected for the different lengths of homology to the exon 4 probe in the 1.25 and 1.7 kb RNAs (see Materials and methods). Since wild-type flies also express a small amount of the 1.9 kb mRNA, the relative level of the truncated *Ifm(3)3* transcript compared with total wild-type Ifm-TmI RNA is actually less than 46%.

A rough correlation between RNA levels and phenotype can be seen by comparing the data in Figure 3A and Table I with the ability of P[TmI,neo]/*Ifm(3)3* heterozygotes to jump and fly (Figure 2, Table I). Line 12-1, with the highest level of RNA expression from the inserted TmI gene, was also the only line which produced a small percentage of fliers when heterozygous with *Ifm(3)3*. Lines 13-2 and 14-1, which produced neither jumpers nor fliers when heterozygous with *Ifm(3)3*, have the lowest levels of Ifm-TmI RNA. It is important to note that *Ifm(3)3* flies contain some Ifm-TmI protein (see below). If this protein is functional, rescue of *Ifm(3)3* may be achieved with lower Ifm-TmI expression from P[TmI,neo] than a completely null Ifm-TmI mutant would require.

The lower panel of Figure 3A illustrates total TmI RNA expression in 1−3-day-old flies. Wild-type and transformed flies contain the abundant 1.3 kb and rarer 1.6 kb Scm-TmI transcripts as well as the 1.7 and 1.9 kb Ifm-TmI RNAs. (The 1.6 kb and much more abundant 1.7 kb RNAs comigrated on the gels used in this study.) The Scm-TmI RNAs also are present in *Ifm(3)3* flies. Accumulation of these transcripts has been shown earlier to be unaffected by the *Ifm(3)3* mutation (Karlik and Fyrberg, 1985). Hybridization in the 1.3 kb region of *Ifm(3)3* and transformant RNA is to both the Scm-TmI RNA and the 1.25 kb *Ifm(3)3* transcript. Since the 1.25 kb transcript appears to be expressed equally in *Ifm(3)3* and transformant RNA (Figure 3A, top panel), we have compared the intensity of hybridization in the 1.25−1.3 kb region of *Ifm(3)3* RNA with that of transformant RNA by densitometry to determine the level of expression of Scm-TmI RNA from P[TmI,neo] (Table I). The different transformants over-express the 1.3 kb Scm-TmI transcript by an amount that roughly parallels their expression of the 1.7 kb Ifm-TmI RNA. These results suggest that the inserted TmI gene produces Scm-TmI as well as Ifm-TmI transcripts. In addition, they indicate that expression of Scm-TmI RNA is not limited to the endogenous *Ifm(3)3* level when additional copies of the TmI gene are present.

The inserted TmI gene appears to respond to the same developmental cues as the wild-type TmI gene. Figure 3B shows that the level of all TmI RNAs decreases dramatically in wild-type, *Ifm(3)3* and transformed lines 12-1 and 13-2 by 9−12 days after eclosion. Ifm-TmI transcripts were not detectable even after long exposure of the blot shown in the top panel of Figure 3B, and Scm-TmI transcripts were much less abundant in older flies (bottom panel). Thus, transcription of both the Ifm-TmI and Scm-TmI RNAs from the inserted TmI gene is subject to normal down-regulation in adults. During embryonic development, the wild-type TmI gene is regulated to produce only Scm-TmI RNA. The 1.3 and 1.6 kb transcripts begin to accumulate in 9-h embryos and increase in abundance through late embryogenesis (Bautch *et al.*, 1982; Basi *et al.*, 1984). To investigate expression of the inserted TmI gene in embryos, blots of total RNA from 1−13-h and 12−24-h wild-type, line 11-3 and line 13-2 embryos were hybridized with either the exon 4 or whole TmI probes. With the exon 4 probe no Ifm-TmI transcripts were detected in wild-type or transformed embryos at either stage. The whole TmI probe revealed that the 1.3 and 1.6 kb Scm-TmI transcripts were present in both wild-type and transformant RNAs (data not shown). We noted above that Scm-TmI transcripts were more abundant in total RNA from transformed compared with wild-type and *Ifm(3)3* flies. Densitometry indicated that the Scm-TmI RNAs were also over-expressed in late embryo RNA from transformed lines 11-3 and 13-2. The 1.6 kb Scm-TmI RNA was 1.5−1.6 times more abundant in late embryo RNA from these two lines than from wild-type embryos. These results suggest that the inserted TmI gene is transcribed in transformed embryos and that the transcripts are spliced correctly.

To determine whether the dosage compensation observed in 10-2 males based on their ability to fly is manifested at the RNA level, we compared TmI RNA expression in 0−3-day-old wild-type and 10-2 females and males (Figure 3C, Table I). Densitometry of various exposures of the RNA blot shown in the top panel of Figure 3C indicated that 10-2 females have 73% of the level of the 1.7 kb Ifm-TmI RNA found in wild-type females. Because histone RNA levels differ significantly between female and male flies, the 1.25 kb *Ifm(3)3* transcript was used as a standard to compare the amount of 1.7 kb RNA in 10-2 females and males. We determined that 10-2 males have 60% of the 1.7 kb RNA present in 10-2 females. This finding combined wih the flight test results indicates that only partial dosage compensation of the inserted TmI gene occurs in 10-2 males.

We next examined the tissue-specificity of RNA expression from the inserted TmI gene. Since all indirect flight muscle is found in the thorax, we compared levels of Ifm-TmI transcripts in the RNA from separated thoraces and abdomens from 2−6-day-old flies (Figure 4A and B, respectively). It can be seen that the wild-type 1.7 kb and 1.9 kb Ifm-TmI mRNAs missing from *Ifm(3)3* thorax are restored in transformed lines 11-3 and 12-1

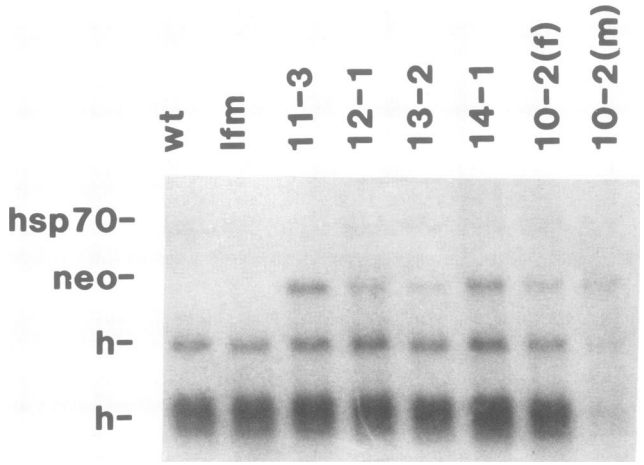

Fig. 5. *Neo* RNA expression in transformed flies. Eight micrograms of total RNA from 1−3-day-old (or 0−3-day-old in the case of 10-2) flies were electrophoresed and blotted as described in the legend to Figure 3. The blot was hybridized with pUC13hsneo and the histone gene clone. The positions of the hsp70, *neo* and histone (h) transcripts are indicated.

(Figure 4A, top panel). No Ifm-TmI transcripts can be detected in the abdominal RNA of wild-type, *Ifm(3)3*, or transformed flies, even after long autoradiographic exposures (Figure 4B, top panel). Thus, Ifm-TmI expression from the TmI gene in P-[TmI,neo] has the tissue-specificity of the wild-type TmI gene. As discussed above, the amount of Scm-TmI RNA in whole fly RNA is greater in the transformants than in *Ifm(3)3* flies (Figure 3A, lower panel). Scm-TmI transcripts also appear to be more abundant in abdominal RNA from transformants (Figure 4B, lower panel), indicating that the TmI gene in P[TmI,neo] is transcriptionally active in abdomen and appropriately using the Scm splicing pathway.

We have examined the uninduced expression of the *neo* gene in adult transformants by probing blots of total RNA from 1−3-day-(or 0−3-day in the case of line 10-2 RNA)-old flies with the P-element vector pUC13hsneo, which contains part of the untranslated leader sequence of hsp70 mRNA as well as the *neo* gene (Figure 1; Steller and Pirrotta, 1985). Levels of *neo* RNA in lines 11-3 through 14-1 were quantitated by densitometry. Results are shown in Figure 5 and Table I. The equally low levels of hsp70 RNA found in each of these samples confirmed that we were assaying uninduced *neo* expression. It can be seen that the level of *neo* RNA varies among the different transformed lines but not necessarily in the way we would predict based on the growth properties of the line on medium containing G418. Line 11-3, which grows well in the presence of drug, has a relatively high level of *neo* RNA (arbitrarily set at 100% in Table I). Line 12-1, which grows poorly on selective medium, has about one-half the *neo* RNA found in line 11-3. Although we did not include the RNA from line 10-2 in our densitometric analysis, this line also has low levels of *neo* RNA and grows poorly on medium containing G418. The unexpected results were manifested by lines 13-2 and 14-1. Line 13-2 grows well on selective medium but has only 41% of the *neo* RNA found in line 11-3. Line 14-1, which is heterozygous for P[TmI,neo] and grows very poorly on G418, has about 78% of the *neo* RNA found in homozygous line 11-3. We also note that *neo* RNA expression does not necessarily parallel expression of the TmI gene insert in the same transposon. For example, line 12-1 expresses low levels of *neo* RNA but high levels of TmI RNA (Figure 3A and Table I). Furthermore, a comparison of *neo* and Ifm-TmI RNA expression in 10-2 females and males shows that while 10-2 males have less Ifm-TmI RNA than 10-2 females (Figure 3C, top panel), *neo* RNA levels appear equal in the two sexes. This suggests that dosage compensation of the *neo* gene in line 10-2 is more complete than that of the TmI gene.

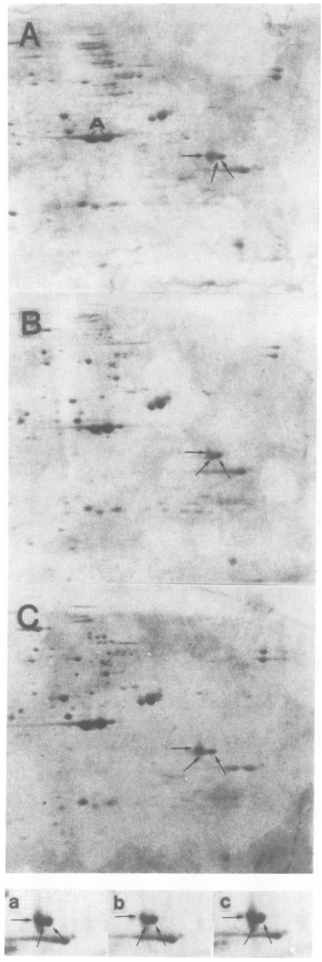

Fig. 6. Two-dimensional gel electrophoresis of total thoracic protein. Thoraces were dissected from 1-day-old wild-type flies **(panel A)**, *Ifm(3)3* flies **(panel B)**, and 11-3 homozygotes **(panel C)**. The gels were stained with Coomassie Blue. The basic end of the gels is to the left; the acidic end is to the right. The second dimension electrophoresis is downward. The A in **panel A** points to the isoforms of actin. The arrows indicate tropomyosin isoforms. Horizontal arrow, Ifm-TmI; left upward pointing arrow, Scm-TmI; right upward pointing arrow, TmII. The bottom panels **(a, b** and **c)** show the tropomyosin region of gels containing twice the amount of protein loaded on the gels in **A, B** and **C**. In **panels A, a**, and **C, c**, the Scm-TmI spot is obscured by the large amount of Ifm-TmI.

Analysis of tropomyosin isoforms in transformed flies

We have confirmed the presence of increased amounts of Ifm-TmI protein in the transformants using two-dimensional gel electrophoresis. Panels A, B and C of Figure 6 display the proteins from Canton S, *Ifm(3)3*, and homozygous line 11-3 thoraces. The bottom three panels (a, b and c) show the tropomyosin region of gels containing twice the amount of protein loaded on the gels in A, B and C. The three muscle tropomyosin isoforms in the

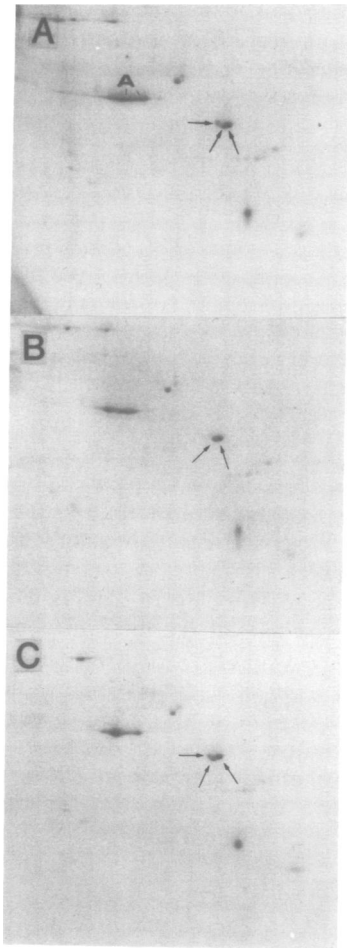

Fig. 7. Two-dimensional gel electrophoresis of total leg protein. Legs were dissected from 1-day-old wild-type **(panel A)**, *Ifm(3)3* **(panel B)**, and 11-3 homozygotes **(panel C)**. Electrophoresis and symbols are described in the legend to Figure 6.

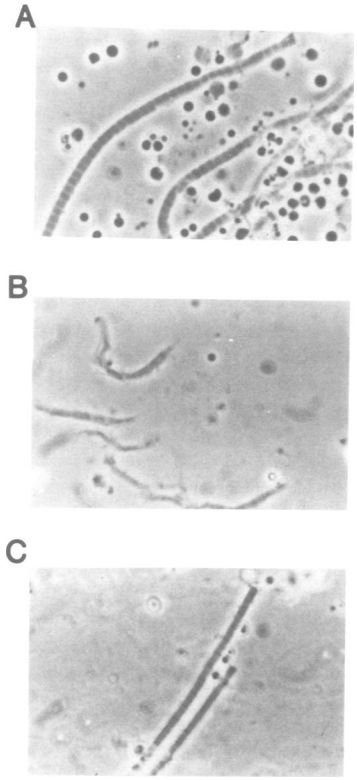

Fig. 8. Phase contrast micrographs of indirect flight muscle myofibrils. Myofibrils were dissected from thoraces of wild-type **(A)**, *Ifm(3)3* **(B)** and transformed line 11-3 **(C)** flies. The samples were viewed at ×1000 magnification.

thorax have previously been identified by protein purification and immunoprecipitation with anti-tropomyosin antibody (Bautch *et al.*, 1982; Basi *et al.*, 1984; R.V.Storti, unpublished observations). The relative amounts of the two TmI isoforms in different samples can be estimated by comparison with the amount of muscle tropomyosin II (TmII) (Figure 6, right upward pointing arrow) which was shown to be unaffected by the *Ifm(3)3* mutation (Mogami and Hotta, 1981; Figure 6B). Wild-type amounts of TmII are also present in line 11-3 (Figure 6C). Approximately 80% of the wild-type thorax consists of indirect flight muscle, and Ifm-TmI is the most abundant thoracic tropomyosin isoform (Figure 6A, horizontal arrow). A small amount of Scm-TmI isoform (Figure 6A, left upward pointing arrow) is present in thorax, but is not well-resolved in this figure because of the large amount of Ifm-TmI. As seen in Figure 6B, and previously shown by Mogami and Hotta (1981), *Ifm(3)3* mutants have reduced amounts of Ifm-TmI. In transformed line 11-3, Ifm-TmI is increased to approximately wild-type levels (Figure 6C). No differences in the amount of Scm-TmI were detected in wild-type, *Ifm(3)3*, and 11-3 samples. We have examined the thoracic proteins of all transformed lines except 14-1, and find that each line has an increased amount of Ifm-TmI compared with the *Ifm(3)3* mutant level (data not shown). The increase in Ifm-TmI was the only consistently observed difference in the two-dimensional gel patterns of *Ifm(3)3* and transformant thoraces. Rescue of the flightless phenotype thus correlates with the synthesis of Ifm-TmI protein.

Since the defect in the jumping ability of *Ifm(3)3* flies can be corrected by P[TmI,neo], we compared the leg proteins of wild-type, *Ifm(3)3*, and transformed line 11-3 flies on two-dimensional gels. Figure 7A shows that wild-type legs contain small amounts of Ifm-TmI (horizontal arrow), in addition to Scm-TmI and TmII. We also have observed low levels of Ifm-TmI RNA in wild-type leg RNA (M.Boardman and R.V.Storti, unpublished observations). In contrast, *Ifm(3)3* legs have little if any, Ifm-TmI protein (Figure 7B). Ifm-TmI is found in the legs of Canton S/*Ifm(3)3* heterozygotes, although it is less abundant than in the legs of Canton S homozygotes (data not shown). Figure 7C shows that approximately wild-type levels of Ifm-TmI are restored to the legs of transformant 11-3 homozygotes. These results indicate that the Ifm-TmI isoform is necessary for the proper function of the leg muscles involved in jumping, and that the inserted TmI gene in line 11-3 is expressed in both leg and indirect flight muscle.

Analysis of myofibrils in transformed flies

We have compared the structure of myofibrils from Canton S, *Ifm(3)3* and line 11-3 indirect flight muscle by phase contrast microscopy (Figure 8). Long, blunt-ended myofibrils with well-defined sarcomeres are abundant in wild-type thorax (Panel A). Myofibrils are scarce in the *Ifm(3)3* thorax (panel B). The few that can be found are easily stretched and torn, so that following

dissection they appear short and irregular in size and shape. The ends of these fibrils are frayed rather than blunt. Sarcomeres are discernable but they have an irregular periodicity (Mogami and Hotta, 1981; Karlik and Fyrberg, 1985). In transformed line 11-3 (panel C) wild-type myofibrillar morphology is almost completely restored. The one difference we note is the smaller diameter of transformant compared with wild-type myofibrils. This may result from slightly reduced levels of Ifm-TmI expression in line 11-3 compared with the wild-type stock, or from differences in the genetic backgrounds of the *Ifm(3)3* and Canton S strains used in this study.

Discussion

Studies employing P-element-mediated transformation of the germ line have had a variety of objectives: to analyse the influence of chromosomal position on gene expression (Spradling and Rubin, 1983; Clark and Chovnick, 1986), to localize sequences involved in DNA amplification (de Cicco and Spradling, 1984; Orr-Weaver and Spradling, 1986) and chromosome puffing (Simon *et al.*, 1985), and to map the regulatory sequences required for proper gene expression (Hiromi *et al.*, 1985; Levis *et al.*, 1985b; Pirrotta *et al.*, 1985; Cohen and Messelson, 1985; Bourouis and Richards, 1985; Fischer and Maniatis, 1986; Garabedian *et al.*, 1986). The availability of a variety of flightless mutants (Koana and Hotta, 1978; Mogami and Hotta, 1981; Deak *et al.*, 1982) makes analyses of the function of myofibrillar proteins and the regulation of their respective genes particularly accessible. Recently, regulatory domains of the *act88F* actin gene and several mutant alleles of *act88F* have been characterized in studies employing P-element transformation (Geyer and Fyrberg, 1986; Mahaffey *et al.*, 1985; Hiromi *et al.*, 1986). We have taken advantage of the *Ifm(3)3* mutation and P-element transformation to study tropomyosin gene regulation. The results presented here demonstrate that a 9.5 kb genomic fragment containing the wild-type TmI gene plus 2.5 kb of 5′ flanking and 2 kb of 3′ flanking DNA can rescue the mutant phenotype of *Ifm(3)3* flies. The inserted TmI gene is expressed with the proper developmental and tissue specificity. These conclusions are based on analyses of flight, myofibrillar morphology, and TmI RNA and protein expression in transformed flies. Our results also demonstrate the feasibility of identifying regulatory elements of the TmI gene by introducing *in vitro* mutagenized TmI sequences into *Ifm(3)3* flies.

Rescue of jumping and flight in Ifm(3)3 flies

Ifm(3)3 flies can neither jump nor fly. We have shown that a defect in the TmI gene is most likely responsible for both phenotypes since the amount of Ifm-TmI protein is greatly reduced in the thorax and legs of these flies and transformation with P-[TmI,neo] restores both jumping and flight. Two copies of P-[TmI,neo] per cell are required to restore the ability to fly to most of the individuals in each transformed line we have examined. The *act88F* actin gene and the myosin heavy chain gene also are haplo — insufficient for the formation of functional indirect flight muscle (Mogami and Hotta, 1981; Bernstein *et al.*, 1983; Hiromi and Hotta, 1985). Jumping in *Ifm(3)3* flies can be rescued with only one wild-type copy of the TmI gene per cell, since Canton S/*Ifm(3)3* flies are able to jump yet contain less leg Ifm-TmI protein than wild-type homozygotes. Similarly, transformed lines 11-3 and 12-1 are able to jump with only one copy of P[TmI,neo] per cell. We do not know whether Canton S, 11-3 or 12-1 flies have a greater ability to jump as homozygotes, and thus we cannot conclude that complete rescue of jumping ability is achieved with one copy of the wild-type TmI gene. A differential response of indirect flight and leg muscle to decreased amounts of a contractile protein also has been observed in flies with defective myosin heavy chain genes. These dominant flightless mutations lower the level of myosin heavy chain protein in thorax and leg by similar amounts but have no detectable effect on leg function (Bernstein *et al.*, 1983; Mogami *et al.*, 1986).

In contrast to lines 11-3 and 12-1, lines 13-2 and 14-1 do not jump when heterozygous with *Ifm(3)3*. Although we have not ruled out the possiblity that P[TmI,neo] is completely inactive in the leg muscle of these two lines, it seems more likely that levels of the Ifm-TmI isoform in the legs of heterozygotes are below the threshold required for jumping. Supporting the latter hypothesis are the lower amounts of Ifm-TmI RNA found in lines 13-2 and 14-1 compared with lines 11-3 and 12-1 (Table I) and tests of individual 13-2 homozygotes indicating that these flies jump as well as fly (unpublished observations).

Some mutant alleles of the *act88F* actin gene prevent proper indirect flight muscle formation in the presence of two copies of the wild-type gene (Hiromi *et al.*, 1986). These mutants produce aberrant proteins that are thought to interfere with the assembly of normal myofibrils. We do not know whether a defective tropomyosin is made in *Ifm(3)3* flies. Since these mutants can be rescued by P[TmI,neo], however, they do not produce enough of an abnormal protein to obstruct muscle assembly. We do find a low level of Ifm-TmI protein with the identical electrophoretic mobility as the wild-type Ifm-TmI isoform in *Ifm(3)3* thoraces, confirming Mogami and Hotta's (1981) observation. This protein may represent the accumulated translation product of wild-type Ifm-TmI RNA present in *Ifm(3)3* flies at levels not detectable by Northern blot analysis. Alternatively, the two aberrant Ifm-TmI transcripts of 1.25 and 10 kb found in *Ifm(3)3* flies might be translated. Although the precise structures of these transcripts have not been determined, our preliminary S1 mapping data indicate that the 1.25 kb transcript extends into the 3′ non-coding region of the fourth exon (unpublished observations), and thus may have all the sequence necessary to encode a functional Ifm-TmI protein. Low amounts of functional Ifm-TmI protein may explain the presence in *Ifm(3)3* flies of small numbers of indirect flight muscle myofibrils with some sarcomeric structure.

From an inspection of the flight tester results and the levels of Ifm-TmI RNA in the transformants (Table I), we can get a rough idea of the minimum amount of Ifm-TmI RNA expression from P[TmI,neo] needed to restore jumping and flight to a population of *Ifm(3)3* flies. We assume that transformants heterozygous with *Ifm(3)3* express Ifm-TmI RNA at one-half the level found in homozygous transformants, and that RNA expression from the inserted TmI gene in the legs of transformants is proportional to its expression in total fly RNA. If these assumptions are correct, minimum expression for proper leg function lies between the levels found in heterozygotes of lines 13-2 (31% wild-type expression — do not jump) and 11-3 (36% wild-type expression — do jump). When the level of expression from the inserted TmI gene reaches that found in line 12-1 heterozygotes (43%) a small percentage of the population can fly. Canton S/*Ifm(3)3* heterozygotes do not fly with 50% wild-type TmI expression. This apparent inconsistency with the results obtained for 12-1/*Ifm(3)3* might be explained if the Ifm-TmI protein in *Ifm(3)3* flies is functional. 12-1/*Ifm(3)3* flies have two copies of the *Ifm(3)3* TmI gene per cell while Canton S/*Ifm(3)3* flies have only one *Ifm(3)3* TmI gene. With 72% of wild-type TmI expres-

sion, line 11-3 homozygotes appear to fly as well as the Canton S stock.

Expression of Scm-TmI RNA in the transformants

Because the *Ifm(3)3* TmI genes in the transformants produce wild-type levels of Scm-TmI RNA, we cannot be certain that the P-[TmI,neo] transposon is generating Scm-TmI transcripts. This seems likely, however, by the increased amounts of Scm-TmI RNA found in transformed compared with wild-type and *Ifm(3)3* embryos and adults. The normal amount of product found in flies diploid for genes encoding dopa decarboxylase, xanthine dehydrogenase, actin, and myosin heavy chain also is exceeded when the copy number of these genes is increased above two per diploid genome (Hodgetts, 1975; Spradling and Rubin, 1983; Geyer and Fyrberg, 1986; Bernstein *et al.*, 1983). The idea that Scm-TmI RNA is transcribed from P[TmI,neo] also is supported by the observation that the level of over-expression of Scm-TmI transcripts among different transformed lines closely parallels the variations in amount of Ifm-TmI RNA.

Variations in expression from P[TmI,neo]

The developmental and tissue-specific regulation of the inserted TmI gene appears to be the same in each of the transformed lines we have examined, and thus is not dependent upon the chromosomal position of the gene. Quantitative variations in expression from both the TmI and *neo* genes in P[TmI,neo] are evident among the different lines, however. Although the level of over-expression of Scm-TmI transcripts by the transformants is roughly proportional to their level of expression of Ifm-TmI RNA, TmI and *neo* gene expression do not vary in parallel. A similar observation has been made by Clark and Chovnick (1986), who found no simple correlation between expression from the *rosy* and *1(3)S12* genes contained within the same P-element transposon in different transformed lines. Variations in expression from P-element transposons are commonly observed, and have been proposed to result from position effects at the site of insertion (Goldberg *et al.*, 1983; Scholnik *et al.*, 1983; Spradling and Rubin, 1983; Hazelrigg *et al.*, 1984; Simon *et al.*, 1985; Bourouis and Richards, 1985; Daniels *et al.*, 1986). This hypothesis was proven in two instances by obtaining proper expression of abnormally regulated *white* and *rosy* genes after moving the P-element transposons containing these genes to new chromosomal locations (Levis *et al.*, 1985a; Daniels *et al.*, 1986). Another less probable explanation for the variation we note would be based on the observation that P-element transposons occasionally mutate upon insertion (Daniels *et al.*, 1985, 1986). Such mutations occur at a very low frequency, however, and thus are unlikely to explain the differences we see between each of the transformed lines. A final factor which may influence expression from P-[TmI,neo] is the pUC8 sequence which becomes integrated into the germ line DNA along with the TmI and *neo* genes in the transformants. In transgenic mice, prokaryotic sequences are known to alter expression from adjacent eukaryotic genes (Chada *et al.*, 1985; Shani, 1986). In the present study, however, the distance between the pUC8 sequence and the TmI and *neo* genes is constant in each transformed line. Thus, the pUC8 DNA is probably not responsible for the variability in TmI expression between lines.

Dosage compensation

In *Drosophila*, genes normally located on the one X chromosome of males are dosage compensated to produce the same amount of product found in females with two active X chromosomes. Some autosomal genes inserted into the X chromosome via P-element transformation undergo at least partial dosage compensation (Spradling and Rubin, 1983; Scholnick *et al.*, 1983). It has been noted that dosage compensation of the *rosy* gene, which is normally expressed at relatively low levels, can be almost complete, but that of the very actively transcribed heat shock genes is slight (Spradling and Rubin, 1983; Pauli *et al.*, 1986). Dosage compensation of the dopa decarboxylase gene also is less when the gene is highly active than when it is transcribed at a slower rate (Scholnick *et al.*, 1983). We have observed different degrees of dosage compensation operating on the X-linked TmI and *neo* genes in line 10-2. Dosage compensation of the more highly expressed TmI gene is only partial while that of the *neo* gene appears complete. To determine whether the differences in dosage compensation exhibited by the TmI and *neo* genes are caused by features of the genes themselves, or by a position effect at the particular location of P[TmI,neo] in line 10-2, several different lines carrying X-linked insertions of P[TmI,neo] must be examined.

Use of G418 selection

Our experience with the five transformed lines described in this report and with others obtained in our laboratory using a different pUC13hsneo−TmI construct indicates a few problems with the use of G418 selection. While some transformed lines grow well on G418, others grow moderately to very slowly on the drug. This variability can delay and make tedious the creation of homozygous or balanced stocks. Because of the marginal growth of some lines, it is also possible that we are not recovering all G1 transformants. G418 selection does not appear to bias our recovery of transformants with a range of expression from the TmI gene, however, since a line's growth rate on G418 does not necessarily correspond to its level of TmI gene expression. The ability of a line to grow on G418 also does not always correlate with the uninduced level of *neo* transcripts found in adults. The level of *neo* expression in larvae is most likely the crucial factor determining growth rate under selection, and this may differ from *neo* expression in adults. A final problem to note is illustrated by each line of transformants we have obtained that carries an X-linked insertion of the P[TmI,neo] transposon (see Materials and methods). While equal numbers of female and male progeny were expected to survive when transformed females were crossed with non-transformed males on medium containing G418, actually only a small percentage of the G418-resistant progeny were female. Homozygous transformed females crossed with transformed males yielded equal numbers of surviving daughters and sons. These results suggest that daughters carrying one copy of the transposon are at a disadvantage compared with sons whose X-linked insertion is subject to dosage compensation. Crosses of transformed males with non-transformed females on medium containing G418 always yielded surviving daughters, but we have not compared the number of surviving progeny with the number of eggs laid to determine if the proportion of surviving daughters depends on whether the non-transformed parent is female or male.

Materials and methods

Drosophila strains

Canton S was the wild-type strain. The *Ifm(3)3* mutation was isolated from ethylmethanesulfonate-treated Canton S males and has been described by Mogami and Hotta (1981). *Ifm(3)3* flies were obtained from Y.Hotta and E.Fyrberg. The second and X chromosome balancer strains *CyO; Ifm(3)3/Ifm(3)3* and *FM6; Ifm(3)3/Ifm(3)3*, respectively, were constructed by standard crosses. The *CyO* chromosome was obtained from the strain *CyO; Tm2,Ubx ry⁻/T(2:3)ap^Xa* provided by A.Chovnick. *FM6* was from *y sn³ cor³⁶/FM6; ry⁵⁰⁶/ry⁵⁰⁶* provided by M.Scott. Third chromosome balancer stocks were *MKRS/In(3L)P+In(3R)P18,kar*

ry^{41} *Ubx e*^{4} obtained from A.Chovnick, and §2*v; Df(3L)vi*^{7}, *ru h gl e ca/Tm3, Sb e Ser* from the Mid-America *Drosophila* Stock Center. See Lindsley and Grell (1968) for a description of balancer chromosomes and genetic markers. Flies were reared at 22°C on yeasted Formula 4-24 (Carolina Biological Supply).

Plasmid strains and construction of p13/TmI

The P-element vector pUChsneo (Steller and Pirrotta, 1985) and the helper plasmid phsπ, which contains a P-element transposase gene attached to the hsp70 promoter and lacks one P terminal repeat, were obtained from V.Pirrotta. pUC13hsneo, containing a unique *Sac*I site, was created by replacing the *Eco*RI−*Sal*I polylinker sequence in pUChsneo with the *Eco*RI−*Sal*I region of the pUC13 polylinker (Pharmacia). To construct the transformation vector p13/TmI, TmI sequences derived from the Canton S genomic clone Dm85 (Bautch *et al.*, 1982) were inserted into pUC13hsneo in several steps. Briefly, a *Bgl*II site ~2 kb 3′ of the last exon of the TmI gene was converted to an *Xho*I site; a 9.5 kb fragment extending from the *Sac*I site ~2.5 kb 5′ of the first TmI exon to the artificial *Xho*I site was then inserted between the *Sac*I−*Sal*I sites of pUC13hsneo (Figure 1).

The exon 4 hybridization probe is a 0.8 kb genomic *Taq*I fragment containing the fourth exon of the TmI gene plus parts of its flanking introns cloned into pBR322. The TmI probe containing most of the TmI gene is a 5.4 kb genomic *Bam*HI−*Bgl*II fragment which spans the last four exons of the gene. A histone clone containing one repeat of the five *Drosophila* histone genes was obtained from S.Lindquist.

P-element transformation

Ifm(3)3 embryos were co-injected with 300 μg/ml of p13/TmI DNA and 100 μg/ml phsπ DNA essentially as described by Spradling and Rubin (1982). Injected embryos were reared on grape agar (Elgin and Miller, 1978) at 22°C until eclosion. G0 adults were mated at 25°C with *Ifm(3)3* flies on yeasted Formula 4-24 medium hydrated with water containing 500 μg/ml G418 (Geneticin, Gibco). *Ifm(3)3* flies produced no surviving progeny on this medium. Every 2−3 days until pupariation cultures were heat-shocked at 37°C for 30 min after removing G0 flies. Individual G418-resistant G1 adults were backcrossed to *Ifm(3)3* flies on medium containing G418. Southern blot analysis (Southern, 1975) of restriction endonuclease-digested DNA from surviving G2 adults revealed the number of P[TmI,neo] insertions carried by different transformed lines and ensured that the transposon had not undergone major rearrangements.

Determining chromosome linkage and establishing homozygous lines

Homozygous or balanced strains were established from those lines known to have one copy of the transposon. The location of the P[TmI,neo] insert in transformed line 11-3 was known from *in situ* hybridization to polytene chromosomes (Gall and Pardue, 1971) to be on chromosome 3. This line was made homozygous by crossing to *MKRS/P18*. Lines 12-1 and 13-2 were crossed to both second and third chromosome balancers. With all three lines G418-resistant flies were crossed to the balancer stocks on selective medium. *Cy* or *Sb* progeny were selected and mated on non-selective medium. The wild-type progeny of the latter cross were used to establish homozygous lines. The P[TmI,neo] insertion sites in lines 12-1 and 13-2 were later located by *in situ* hybridization.

X-linkage of the P[TmI,neo] insert in line 10-2 was determined by backcrossing transformed males to *Ifm(3)3* females on G418 medium. From this cross only female progeny survived. When trying to make line 10-2 homozygous we encountered a problem that was subsequently observed in two other transformed lines carrying X-linked insertions of pUC13hsneo plus a modified TmI gene. When transformed females were crossed with non-transformed males on selective medium, a very small percentage of the surviving progeny were female, although equal numbers of males and females were expected. Equal numbers of sons and daughters were produced when transformed females were backcrossed to non-transformed males on non-selective medium. These results were obtained when the X-linked transposon was in the *Ifm(3)3* or P2 (Oregon R) background, and with transformants that grow well or poorly on G418. Because of this problem, line 10-2 was made homozygous by first crossing G418-resistant 10-2 males with *FM6; Ifm(3)3/Ifm(3)3* females. Bar-eyed daughters were then mated with G418-resistant 10-2 males. Female and male progeny with wild-type eyes were used to establish the homozygous stock. Each of the above crosses took place on non-selective medium.

Because line 14-1 produced very few G418-resistant progeny each generation when grown on selective medium, we used the following scheme to balance the line on non-selective medium. One G418-resistant 14-1 male was crossed to females of one of each of the balancer stocks for the second, third and X chromosomes. Approximately 40 F_1 progeny carrying the balancer chromosomes were then crossed in pairs. For each pair cross, F_2 progeny lacking the balancer chromosome were crossed *en masse*. Several pair crosses produced no wild-type progeny suggesting that line 14-1 carried a homozygous lethal mutation. DNA was prepared from each pair-cross line, from non-transformed flies, and from transformants known to contain one and two copies of the P[TmI,neo] insert per diploid genome. The DNA was dotted onto nitrocellulose filters and hybridized with ^{32}P-labelled pBR322. Since P[TmI,neo] included pUC8 sequences that were derived from pBR322, this experiment should indicate the number of copies P[TmI,neo] carried by the pair-cross lines. After comparing the hybridization signal from the pair-cross lines with the standards we found no homozygous lines. Each of the three-pair cross lines arising from the cross to *MKRS* that produced only *Sb* F_2 progeny contained one copy of the pBR322 sequences per diploid genome. Therefore, we tentatively concluded that the P[TmI,neo] insert in line 14-1 is on chromosome 3 and is lethal when homozygous. The location was later confirmed by *in situ* hybridization.

Flight tests

A modified version of the flight tester described by Okamoto *et al.* (1986) was used. It consisted of a covered transparent polycarbonate cylinder (30 × 30 cm) seated on end and marked at 5 cm intervals from top to bottom. A funnel was inserted into the center of the top and extended 9 cm into the cylinder. A 4 cm square plastic dish (the swing) was hung 3 cm below the funnel. Just before a test, the sides of the cylinder were coated with mineral oil. Flies (100−250) (4−7 days old) were gently tapped down through the funnel and onto the swing. After 3 min the number of flies stuck in the oil on the sides and bottom of the cylinder and those remaining on the swing were counted. To compare flight in females and males, flies were lightly anesthetized with triethylamine (Fly Nap, Carolina Biological Supply) 4 days before the test and the sexes were separated.

RNA preparation and analysis

Total RNA was prepared from whole flies (equal numbers of females and males), thoraces and abdomens, or embryos, by precipitation from guanidinium isothiocyanate and guanidine hydrochloride (Chirgwin *et al.*, 1979). RNA was electrophoresed for ~400 volt-hours on 1.5% agarose gels containing formaldehyde, basically as described in Maniatis *et al.* (1982), and then was blotted to nitrocellulose filters overnight. After baking, the filters were prehybridized at 42°C for at least 4 h in 50% formamide, 2.5 × SET (1 × SET: 0.15 M NaCl, 30 mM Tris, 1 mM EDTA, pH 8), 5 × Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 10 μg/ml poly(A), 200 μg/ml denatured salmon testis DNA, and then were hybridized overnight at 42°C in 50% formamide, 2.5 × SET, 5 × Denhardt's solution, 20 mM sodium phosphate, pH 6.5, 10 μg/ml poly(A), 100 μg/ml denatured salmon testis DNA, 8% dextran sulfate, containing 1.5 × 10^6 c.p.m./ml exon 4, whole TmI or pUC13hsneo plasmid DNA probes and 1.5 × 10^5 c.p.m./ml histone plasmid DNA. All probes were labelled with ^{32}P by nick translation (Maniatis *et al.*, 1982). Filters were washed and then autoradiographed using XAR-5 film with or without intensifying screens.

To quantitate the relative amounts of the 1.7 kb Ifm-TmI transcript in wild-type and transformant RNA, the hybridization intensity of that transcript and the largest histone mRNA was determined by scanning various exposures of the blot illustrated in the top panel of Figure 3A with a densitometer. The levels of the 1.7 kb transcript shown in Table I were corrected for differences in the amount of RNA loaded in different lanes by reference to the histone signal. To compare the relative amounts of 1.3 kb Scm-TmI transcript in *Ifm(3)3* and transformant RNA, the hybridization signal in the 1.25−1.3 kb region on various exposures of the blot shown in the bottom panel of Figure 3A was determined by densitometry. Our preliminary S1 mapping experiments indicate that the 1.25 kb *Ifm(3)3* transcript contains approximately the first 208 nucleotides of the 429 bp fourth exon (unpublished observations). All 429 nucleotides of the fourth exon in the exon 4 probe will hybridize with the wild-type 1.7 kb Ifm-TmI RNA, but only 208 nt will hybridize with the 1.25 kb transcript. We corrected for this differential binding when comparing the level of the 1.25 kb transcript in *Ifm(3)3* RNA with the 1.7 kb Ifm-TmI transcript in wild-type RNA shown in the top panel of Figure 3A.

Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis of total thoracic and leg proteins was performed as described previously (Bautch *et al.*, 1982). Thoraces or legs from 1-day-old flies were homogenized in isoelectrofocusing buffer. Total protein from approximately four thoraces or 10 legs was electrophoresed per gel. Gels were stained with Coomassie Blue. To compare the relative amount of tropomyosin in different samples, the amount of protein was standardized against the amount of actin and tubulin.

Preparation of indirect flight muscles

Adult female flies were lightly anesthetized with triethylamine 7−8 days after eclosion. The isolated thorax was extensively teased apart with dissecting needles in a drop of insect Ringers solution (0.13 M NaCl, 5 mM KCl, 2 mM $CaCl_2$). Indirect flight muscle was observed by phase contrast microscopy at ×1000.

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