NOTES

Small Differences in *Drosophila* Tropomyosin Expression Have Significant Effects on Muscle Function

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The effects of promoter deletions on *Drosophila* tropomyosin I (TmI) gene expression have been determined by measuring TmI RNA levels in transformed flies. Decreases in RNA levels have been correlated with rescue of flightless and jumpless mutant phenotypes in *Ifm(3)3* mutant transformed flies and changes in muscle ultrastructure. The results of this analysis have allowed us to identify a region responsible for 20% of maximal TmI expression, estimate threshold levels of TmI RNA required for indirect flight and jump muscle function, and obtain evidence suggesting that sarcomere length may be an important determinant of flight muscle function.

The assembly of thick and thin filaments into functional myofibrils requires the synthesis of both contractile and accessory proteins at the appropriate time and in the correct stoichiometric amounts. Myofibril assembly in *Drosophila melanogaster* is being elucidated through studies of indirect flight muscle (IFM) mutants which have defects in a variety of contractile-protein genes. Molecular genetic analysis of many of these dominant flightless mutants has indicated that myofibril assembly and function are highly sensitive to gene dosage and to the ratio of individual myofibrillar components (2, 4, 5, 11).

In our analysis of tropomyosin gene expression in *D. melanogaster*, we have made use of the dominant flightless tropomyosin mutation, *Ifm(3)3* (11). The mutation is caused by a transposable element insertion into the alternately spliced fourth exon of the tropomyosin I (TmI) gene (7). This exon is normally expressed only in the IFM and jump muscle (tergal depressor of the trochanter [TDT]) of the adult (1). The *Ifm(3)3* mutation severely reduces expression of the IFM-TDT-specific TmI isoform; as a result, homozygous *Ifm(3)3* flies neither jump nor fly. *Ifm(3)3/+* heterozygotes jump but cannot fly. Expression of a second, non-IFM-TDT TmI isoform is unaffected by the *Ifm(3)3* mutation. We demonstrated earlier that a cloned TmI gene introduced into *Ifm(3)3* flies by P-element-mediated transformation restores TmI RNA and protein and the ability to jump and fly to nearly wild-type levels (19). Significantly, two copies of the inserted TmI gene per cell are necessary to restore flight, while one copy of the transgene restores the ability to jump.

Our current investigations focus on identifying TmI promoter elements. Recently we located an enhancerlike region in the first intron of the gene (14). In the present study, we have analyzed the effects of deletions of 5'-flanking DNA on TmI expression and have identified a 229-bp region responsible for stimulating approximately 20% of maximal TmI RNA accumulation. We have also found that relatively small changes in TmI RNA levels have significant effects on muscle function, as judged by the ability of *Ifm(3)3* transfor-

mants to jump and fly. Ultrastructural analysis indicates that increasing the amount of tropomyosin expressed in *Ifm(3)3* IFM causes an increase in myofibril diameter followed by sarcomere elongation. Our results suggest that a small increase in TmI can trigger the transition from flightless to flying phenotype, in part by allowing sarcomeres to reach a critical length at which function is restored.

Effects of 5' deletions on TmI RNA and phenotype. *Ifm(3)3* flies were transformed with a series of TmI genes containing variable amounts of 5'-flanking DNA (Fig. 1). IFM and TDT functions in transformants homozygous for each TmI transgene were determined (Fig. 1), and total TmI RNA expression was measured by primer extension analysis of adult RNA (Fig. 2). The results summarized in Table 1 correlate phenotypes with TmI RNA levels. In these experiments, independent lines of flies transformed with the same construct were grouped by phenotype. RNA was prepared from pools of 100 flies from each transformed line in a group of like phenotype to obtain an average RNA level for a given phenotype.

TmI genes with 2,500, 1,900, or 790 bp of 5'-flanking DNA restored both jumping and flight to 16 of 18 lines (89%) of the *Ifm(3)3* transformants. In lines transformed with the Δ -2500 or Δ -790 TmI genes, the ratio of transgenic RNA expression to endogenous *Ifm(3)3* TmI expression averaged 0.78 ± 0.07 for those lines able to fly. For purposes of comparison, we refer to this as the normal level of transgenic TmI RNA. In Table 1, the ratios of transgenic to endogenous TmI RNA for all other groups of TmI transformants were normalized to the average expression ratio for Δ -2500 and Δ -790 flying transformants.

A TmI gene containing 452 bp of 5'-flanking sequence (Δ -452) restored the ability to jump and fly to only two of five transformed lines. All five lines regained the ability to jump. The average level of transgenic TmI RNA in the five Δ -452 lines was 82% of normal. Thus, an 18% drop in transgenic TmI expression decreased the number of flying transformants to 45% (0.4/0.89) of normal. One explanation for these results is that the deletion to -452 removed elements that specifically enhance IFM TmI expression, causing a large drop in TmI RNA levels in these muscles but no change in TmI expression in other muscles of the fly. Since our RNA assay measures total TmI transcripts, we

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FIG. 1. Phenotypic analysis of *Ifm(3)3* flies transformed with 5' deletions of the TmI gene. The extent of the 5'-flanking region remaining in each construct is shown schematically at the left. (The figure is not drawn to scale.) Boxes represent the first exon and part of the second exon. All constructs contain the complete TmI gene and 2 kb of 3'-flanking DNA. Deletions were generated by BAL 31 digestion of a TmI gene containing a 10-bp *Bam*HI linker inserted at the *Ava*I site in exon 1 (+10) (14). The linker insertion allows us to differentiate endogenous *Ifm(3)3* TmI transcripts from transgenic RNA, which is 10 nucleotides (nt) longer (see Fig. 2). Selected constructs were sequenced to determine the extent of the deletions. The deleted TmI genes Δ-1900, Δ-790, and Δ-452 were inserted between the *Sac*I and *Sal*I sites of the P-element vector pUC13hsneo (17, 19). All other TmI genes were inserted between the *Kpn*I and *Xho*I sites of the P-element vector pW8 (8). Transformations were carried out essentially as described previously (16, 19). *Ifm(3)3* flies obtained from Y. Hotta (11) were the recipients of plasmids derived from pUC13hsneo. Transformants were identified by their resistance to G418. Plasmids derived from pW8 were injected into white *Ifm(3)3* flies; transformants were identified by restoration of eye pigmentation. Blot analysis of genomic DNA prepared from each transformed line was used to determine the copy number and verify the integrity of the transgene. Only homozygous transformants containing a single, unrearranged copy of the transgene per haploid genome were analyzed in this study. In previous experiments, a flight tester was used to quantitate rescue of the *Ifm(3)3* phenotype (19). Because of the large number of transformants generated in the experiments whose results are presented here, it was impractical to measure flight for all transformants by using the flight tester. Accordingly, phenotypic analysis was qualitative. Transformed lines were judged en masse to be capable of either jumping or flight. Although subtle differences exist among lines with similar recorded phenotypes, no attempt was made to categorize these distinctions in this report.

therefore detect a relatively small decrease in total TmI RNA. Alternatively, transgenic TmI expression could decrease by 18% in the Δ-452 transformants. Experiments described elsewhere (14) suggest that expression more than likely is uniformly reduced in all muscle. Notable sequences in the region between positions -790 and -452 whose loss might account for reduced TmI expression include a 27-bp stretch of alternating AT residues and numerous short direct repeats (1). Similar repeating elements are also found in the 5'-flanking DNA of the *Drosophila* 79B and 88F actin genes (13). Our results suggest that any effect on TmI expression by specific sequences within the region accounts for less than 20% of maximal TmI transgene transcription.

The TmI gene containing only 146 bp of 5'-flanking DNA (Δ-146) failed to restore flight to any of the five transformed lines we obtained. Only one of the lines was able to jump. Despite the low level of phenotypic rescue, average transgenic RNA production in these five lines was 43% of normal. Thus, a TmI gene containing only 146 bp of 5'-flanking DNA still produced a significant amount of properly initiated TmI RNA, although not enough to restore IFM function and only marginally able to support TDT function. The element(s) responsible for accurate initiation of transcription must be present in the Δ-146 gene. The TmI gene does not contain a TATA box at the usual position 25 bp 5' of the start of transcription. Other sequences which may contribute to expression in this construct are a CAAT element at position -118 and multiple runs of TGs (1).

The decrease in TmI expression in the Δ-452 and Δ-146 transformants might be due to either the loss of promoter elements contained within the deleted regions described above or the encroachment of inhibitory P element or

neighboring chromosomal sequences on the 5' end of the TmI gene (12). To avoid this ambiguity in our next series of experiments, we tested the effects of three small deletions in which the bulk of 5'-flanking DNA was retained (Fig. 1). We focused on deletions in the region between positions -374 and -146, since this stretch of sequence contains four short elements similar to sequences present in the 5'-flanking DNA of the *Drosophila* tropomyosin II (TmII) or actin^{88F} gene (see Fig. 5 in reference 6). The largest deletion, from -374 to -146 [Δ(-374-146)], removes all of these elements. The Δ(-374-146) TmI gene rescued flightlessness in four of seven lines and the inability to jump in six of seven lines. The average level of transgenic TmI RNA in these seven lines was 83% of the level in the Δ-2500 and Δ-790 transformants. The construct Δ(-194-146) has lost the region of similarity with the actin^{88F} gene but retains the TmII-like elements. This deletion had minor consequences: all Δ(-194-146) transformants could fly, and RNA levels were 91% of the normal level. Only one transformed line was obtained with the Δ(-210-146) TmI gene; this line was able to jump and fly. In the absence of additional Δ(-210-146) transformants, we conclude that the two regions, -374 through -195 and -194 through -146, each contain promoter elements responsible for approximately 10% of maximum TmI expression.

Threshold levels of transgenic TmI required for IFM and TDT functions. Comparison of phenotype with TmI RNA expression in the transformed lines in Table 1 supports our earlier observation that IFM requires more TmI than does jump muscle to function (19). Thus, in the present study, an average transgene expression ratio of 0.69 was the lowest we

FIG. 2. Primer extension analysis of RNAs from flies transformed with 5' deletions of the TmI gene. First lane, RNA from nontransformed control flies; second lane, RNA from flies transformed with a TmI gene which does not include the *Bam*HI linker in the first exon (19); remaining lanes, RNA from the transformants indicated in Table 1. N (jumpless and flightless), J (jump), and F (jump and fly) indicate the observed phenotypes. The primer (P), endogenous (E), and transgenic (T) TmI RNA extension products are also indicated. Total RNA was prepared from 1- to 2-day-old whole flies as described elsewhere (3). Primer extension analysis was carried out as described previously (10, 14). Extensions were initiated from a 23-nt primer complementary to the 5' end of the second exon (+2163 to +2185). The reaction products were recovered by ethanol precipitation and analyzed by electrophoresis on 8% polyacrylamide-8.3 M urea sequencing gels, followed by autoradiography of the gel. The amount of extended product was measured by excising the appropriate bands (region T bands plus the additional larger bands; see below) from the gel and measuring the radioactivity in a scintillation counter. Transcription from the endogenous TmI gene initiates at a cluster of three sites (1). Products extended from endogenous *Ifm(3)3* TmI RNA are 80, 81, and 83 nt long (region E). Transgenic TmI RNA, which is 10 nt longer than the endogenous RNA because of the *Bam*HI linker insertion, also initiates at three sites. Most of the transgenic RNA extension products are contained in bands at 90, 91, and 93 nt (region T). RNA from transformants generates a series of larger minor products in addition to the expected products. These additional bands were never observed for RNA extracted from either nontransformed lines [white; *Ifm(3)3*] or lines transformed with a TmI gene without the linker [Δ -2500(10-2)]. It is possible that proper positioning of RNA polymerase II at the normal start site was sometimes disrupted by the linker insertion, leading to the observed extraneous sites of initiation.

TABLE 1. TmI RNA expression in *Ifm(3)3* flies transformed with 5' deletions of the TmI gene

Transgene	Phenotype ^a	Transgenic TmI/ endogenous TmI ^b $\pm$ SD	Weighted avg ^c $\pm$ SD	Normalized ratio ^d
Δ -2500	F (7)	0.79 $\pm$ 0.04		
Δ -790	F (3)	0.76 $\pm$ 0.16	0.78 $\pm$ 0.07	1.00
Δ -452	F (2)	0.78 $\pm$ 0.04		
Δ -452	J (3)	0.54 $\pm$ 0.09	0.64 $\pm$ 0.07	0.82
Δ -146	J (1)	0.56 $\pm$ 0.04		
Δ -146	N (4)	0.29 $\pm$ 0.12	0.34 $\pm$ 0.10	0.43
Δ -(374-146)	N (1)	0.56 $\pm$ 0.07		
Δ -(374-146)	J (2)	0.61 $\pm$ 0.04		
Δ -(374-146)	F (4)	0.69 $\pm$ 0.05	0.65 $\pm$ 0.04	0.83
Δ -(194-146)	F (10)	0.72 $\pm$ 0.04		0.91

^a Phenotypes: N, jumpless and flightless; J, jump; F, fly and jump. The numbers in parentheses are the total number of lines which share that phenotype.

^b Each ratio represents the average expression level ratio calculated from two separate determinations. Expression levels were determined by cutting out the appropriate bands from the gels (see Fig. 2 legend), measuring the radioactivity, subtracting background, and expressing the ratio of transgenic TmI counts to endogenous TmI counts.

^c Weighted averages represent the average RNA expression ratios for the indicated phenotypes.

^d Normalized to values for Δ -2500 and Δ -790, i.e., (0.79 + 0.76)/2 = 0.78.

observed for pools of flying strains, while a ratio of 0.54 was the lowest we observed for pools of jumpers.

To determine approximate threshold levels of transgenic TmI RNA required for jumping and flight, we examined individual lines of transformants which showed a range of phenotypes but differed relatively little in their levels of transgenic TmI expression (Table 2). Of the four lines of Δ -(374-146) transformants able to both fly and jump, lines D and F had the lowest transgenic TmI expression ratio (0.65) that still allowed flight. Three individual lines of flies which could jump but not fly were examined. Line Δ -(374-146)_C had the highest level of TmI expression (0.61). Thus, the threshold transgenic TmI expression ratio required to restore flight to the *Ifm(3)3* strain is between 0.61 and 0.65. Two lines of transformants listed in Table 2 had a transgenic TmI

TABLE 2. TmI RNA expression in independent lines transformed with Δ -(374-146)

Transgene	Phenotype ^a	Transgenic TmI/ endogenous TmI ^b $\pm$ SD	Normalized ratio ^c
Δ -(374-146) _L	F	0.75 $\pm$ 0.04	0.96
Δ -(374-146) _H	F	0.69 $\pm$ 0.01	0.88
Δ -(374-146) _D	F	0.65 $\pm$ 0.04	0.83
Δ -(374-146) _F	F	0.65 $\pm$ 0.01	0.83
Δ -(374-146) _C	J	0.61 $\pm$ 0.03	0.78
Δ -(374-146) _E	J	0.60 $\pm$ 0.03	0.77
Δ -(374-146) _B	N	0.56 $\pm$ 0.07	0.72

^a Phenotypes: N, jumpless and flightless; J, jump; F, fly and jump.

^b Each ratio represents the average expression level ratio calculated from two separate determinations. Expression levels were determined by cutting out the appropriate bands from the gels (see Fig. 2 legend), measuring the radioactivity, subtracting background, and expressing the ratio of transgenic TmI counts to endogenous TmI counts.

^c Normalized to Δ -2500 and Δ -790, i.e., 0.78 = 1.00 (Table 1).

FIG. 3. Ultrastructure of IFM in wild-type, *Ifm(3)3*, and Δ -(374-146) transformant flies. Thoraces from 5- to 8-day-old females were fixed in 6.25% glutaraldehyde-0.125 M sodium cacodylate (pH 7.2), postfixed in 1% osmium tetroxide, and embedded in LX-112 by standard procedures. We confirmed before fixation that each of the Δ -(374-146)_E transformants was unable to fly but able to jump; each of the Δ -(374-146)_H transformants was able to fly. Straw-gold sections were stained with 5% uranyl acetate and 0.2% lead citrate. Dorsal longitudinal IFM was examined in cross section (a through d) and longitudinal section (e through h). Panels a and e, wild type (M, M band; Z, Z band) panels; b and f, *Ifm(3)3*; panels c and g, Δ -(374-146)_E. Arrows in panel g point to Z- and M-band defects. Panels d and h, Δ -(374-146)_H. Arrows in panel h point to abnormal shifts in the position of the M band. Panels a through d, bar = 0.5 μ m; panels e through h, bar = 1 μ m.

expression ratio of 0.56; only one of these strains could jump. A pool of three jumping lines transformed with the Δ -452 TmI gene had a ratio of 0.54 (Table 1). Thus, the minimal transgenic TmI expression ratio that allows flies to jump is probably close to 0.54. It should be noted that *Ifm(3)3* is not a null mutation. We and others have observed small amounts of a polypeptide with the same electrophoretic mobility as the IFM-TDT-specific TmI isoform in *Ifm(3)3* flies (11, 19). The endogenous *Ifm(3)3* RNA encoding the IFM-TDT-specific protein is expressed at approximately 40% of the level of wild-type IFM-TDT TmI RNA (19). To estimate the minimum amount of TmI RNA required by indirect flight and jump muscles, the contribution from the *Ifm(3)3* TmI gene must be included. The total TmI RNA measured in this study includes transcripts for both the IFM-TDT and the non-IFM-TDT isoforms. Total TmI RNA in 1- to 3-day-old *Ifm(3)3* flies is approximately one-half that in wild-type flies (18). Thus, a homozygous transformant with a transgenic/endogenous *Ifm(3)3* expression ratio of 0.65 is producing transgenic TmI RNA at 33% of the wild-type level. Adding this to the TmI RNA contributed by the *Ifm(3)3* TmI gene (40% of the wild-type level) yields a requirement for approximately 73% of wild-type expression for flight. A transformant with a transgenic/endogenous *Ifm(3)3* expression ratio of 0.54 is expressing transgenic TmI RNA at 27% of the level of wild-type flies. Assuming that transgenic TmI expression in jump muscle is supplemented by transcripts from the *Ifm(3)3* TmI gene at 40% of wild-type levels, jump muscle is disabled when TmI RNA falls below about 67% of wild-type levels. Thus, IFM tolerates a 27% decline in TmI expression, while jump muscle accommodates a slightly larger drop (33%), before function is lost. The defects in muscle function may be caused by failure to meet a strict requirement for a high level of tropomyosin. Alternatively, they may result from a stoichiometric imbalance in the levels of tropomyosin and other contractile proteins as tropomyosin production falls. The latter effect has been seen in certain actin and myosin mutants (2).

One additional consideration from these results relates to the use of reporter genes such as the *lacZ* gene (9) to study the effects of DNA deletions on gene expression. It is clear from the work reported here that deletions which result in only a small decrease in mRNA can have profound physiological consequences. Since small differences in expression are not readily detected in typical reporter gene assays, caution is needed when interpreting the results of deletion studies employing reporter genes.

Flight muscle ultrastructure. To determine whether the transition from flightless to flying behavior caused by a small increase in TmI expression is associated with changes in flight muscle morphology, we compared IFM ultrastructure in transformed lines Δ -(374-146)_E and Δ -(374-146)_H. Flies in line Δ -(374-146)_E are flightless (transgenic TmI expression ratio, 0.60), while those in line Δ -(374-146)_H are able to fly (transgenic TmI expression ratio, 0.69) (Table 2). Cross sections of IFM show that thick and thin filaments are packed in a double hexagonal array in wild-type myofibrils; in the *Ifm(3)3* strain, only the filaments in the core of each fibril are so arranged (Fig. 3a and b) (7, 15). While the double hexagonal lattice is much more extensive in both transformed lines than in the untransformed mutant, filament packing is often interrupted along the outer margins of the fibrils in the transformants (Fig. 3c and d). Thus, most fibrils contain peripheral patches of normally packed filaments offset from the larger core lattice. Fibrils from the two transformants are about equal in diameter. At their widest,

wild-type myofibrils contain about 37 thick filaments. The widest fibrils from both line Δ -(374-146)_E and line Δ -(374-146)_H are larger than wild-type fibrils, occasionally having 40 or more thick filaments.

In cross section, myofibrils from lines Δ -(374-146)_E and Δ -(374-146)_H are indistinguishable, but differences between the two transformed lines become apparent when longitudinal sections are examined (Fig. 3e through h). Wild-type myofibrils are straight, with regularly spaced Z and M bands that extend the width of the fibril; each sarcomere is approximately 3.4 μ m long. *Ifm(3)3* fibrils are wavy, the Z and M bands vary in size and are regularly spaced only in the center of each fibril, and the average sarcomere is 2.7 μ m long. Myofibrils from the two transformed lines are much improved compared with those of the mutant, although they still display a range of defects (e.g., branching peripheral filaments and disrupted Z and M bands). The defects are generally more severe in flightless line Δ -(374-146)_E. Examples of better-organized fibrils from the transformants are shown in Fig. 3g and h. Δ -(374-146)_H fibrils are straighter and contain longer sarcomeres than Δ -(374-146)_E fibrils. An average sarcomere is 3.2 μ m long in line Δ -(374-146)_H but only 2.7 μ m long in line Δ -(374-146)_E. Thus, the increase in TmI expression in line Δ -(374-146)_E over that of *Ifm(3)3* flies widened the diameter of the myofilament lattice to about that in line Δ -(374-146)_H but did not change the length of the sarcomere. Our results suggest that the small increase in TmI expression in line Δ -(374-146)_H compared with that of line Δ -(374-146)_E allowed sarcomeres to surpass a critical length beyond which function is restored. Alternatively, function may be restored by some other feature of Δ -(374-146)_H muscle, e.g., decreased myofilament packing abnormalities. Sarcomere lengthening might then be an effect rather than a cause of rescued function.

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