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A Muscle-Specific Intron Enhancer Required for Rescue of Indirect Flight Muscle and Jump Muscle Function Regulates *Drosophila* Tropomyosin I Gene Expression

JOSHUA R. SCHULTZ,[1] TERESE TANSEY,[2] LINDA GREMKE,[1] AND ROBERT V. STORTI[1]*

Department of Biochemistry, University of Illinois College of Medicine, Chicago, Illinois 60612,[1] and Department of Biology, Georgetown University, Washington, D.C. 20057[2]

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The control of expression of the *Drosophila melanogaster* tropomyosin I (TmI) gene has been investigated by P-element transformation and rescue of the flightless and jumpless TmI mutant strain, *Ifm(3)3*. To localize *cis*-acting DNA sequences that control TmI gene expression, *Ifm(3)3* flies were transformed with P-element plasmids containing various deletions and rearrangements of the TmI gene. The effects of these mutations on TmI gene expression were studied by analyzing both the extent of rescue of the *Ifm(3)3* mutant phenotypes and determining TmI RNA levels in the transformed flies by primer extension analysis. The results of our analysis indicate that a region located within intron 1 of the gene is necessary and sufficient for directing muscle-specific TmI expression in the adult fly. This intron region has characteristics of a muscle regulatory enhancer element that can function in conjunction with the heterologous nonmuscle *hsp70* promoter to promote rescue of the mutant phenotypes and to direct expression of an *hsp70-Escherichia coli lacZ* reporter gene in adult muscle. The enhancer can be subdivided further into two domains of activity based on primer extension analysis of TmI mRNA levels and on the rescue of mutant phenotypes. One of the intron domains is required for expression in the indirect flight muscle and jump muscle of the adult. The function of the second domain is unknown, but it could regulate the level of expression or be required for expression in other muscle.

Myogenesis, the formation of muscle from its mesodermal origin, is brought about by the transcriptional activation of sets of muscle-cell-specific genes at both the determination and differentiation phases of myogenesis (15, 17–19, 34, 37, 39, 40, 43, 53). This later step is characterized by the coordinated expression of the major contractile muscle proteins, including the myosin heavy and light chains, cardiac and skeletal actins, tropomyosin, and troponin (2, 5, 9, 11, 27, 46). Molecular approaches have been used to reveal that the transcriptional control of a number of vertebrate muscle genes is regulated by *cis*-acting DNA sequence elements located 5′ and 3′ to the genes as well as within the genes. Many of these control regions have been shown to interact with protein factors that may be involved in their expression. Most *cis*-acting transcription elements have been identified by introducing mutations in vitro into cloned muscle genes, transfecting the mutated genes into tissue culture muscle cell lines that simulate the latter stages of the myogenic program and analyzing their effects on expression. Multicellular organisms, however, are made up of several different muscle cell types, all of which are influenced throughout development by inductive interactions, nervous stimulation and trophic factors that regulate their differentiation. The analysis of expression in tissue culture therefore subjects the transfected genes to only a single cell type having a more limited repertoire of regulatory signals than what is typically seen in vivo. This could reflect artifacts of muscle gene regulation.

Myogenesis in *Drosophila melanogaster*, on the other hand, is similar to muscle development in vertebrates and provides an excellent model system in which to study muscle gene regulation in the whole organism (14). A large number of genetic lesions have been characterized in *D. melanogaster* that affect myofibrillar protein synthesis, assembly, and function in muscles that are dispensable for viability and reproduction (6, 16, 38). These studies have indicated that muscle gene regulation is subject to a complex pattern of controls that integrate with other muscle genes. The development of P-element germ line transformation (49) has provided the opportunity to study the function of DNA regulatory elements by insertion of single copies of cloned genes into germ line chromosomes. In contrast to studies done by using tissue culture, these in vivo transformations permit the study of cloned genes throughout development in all cells of the organism and in different muscle mutant backgrounds.

In this study, we have combined P-element transformation and rescue of tropomyosin mutant phenotypes to identify *cis*-acting sequences controlling expression of the *Drosophila* tropomyosin I (TmI) gene. In previous work we characterized the structure and expression of the TmI gene (3–5). It was demonstrated that the TmI gene encodes two muscle-specific tropomyosin isoforms by alternately splicing exon 4 of the gene, thereby generating two sets of tissue-specific mRNA transcripts (3). Larval and adult visceral and somatic tubular muscle produce transcripts consisting of exons 1 to 3 and 5. An alternate set of transcripts, consisting of exons 1 to 5, is expressed in thoracic indirect flight muscle (IFM) and in the jump muscle of the leg (tergal depressor of the trochantor or TDT) in adults. Both mRNAs encode 34,000-Da muscle tropomyosin isoforms which differ in their last 27 amino acids. The *Ifm(3)3* mutation is a tropomyosin mutation that affects tropomyosin expression in IFM and TDT muscle. The mutation results from an 8.8-kb transposable element insertion within the alternately spliced exon 4

* Corresponding author.

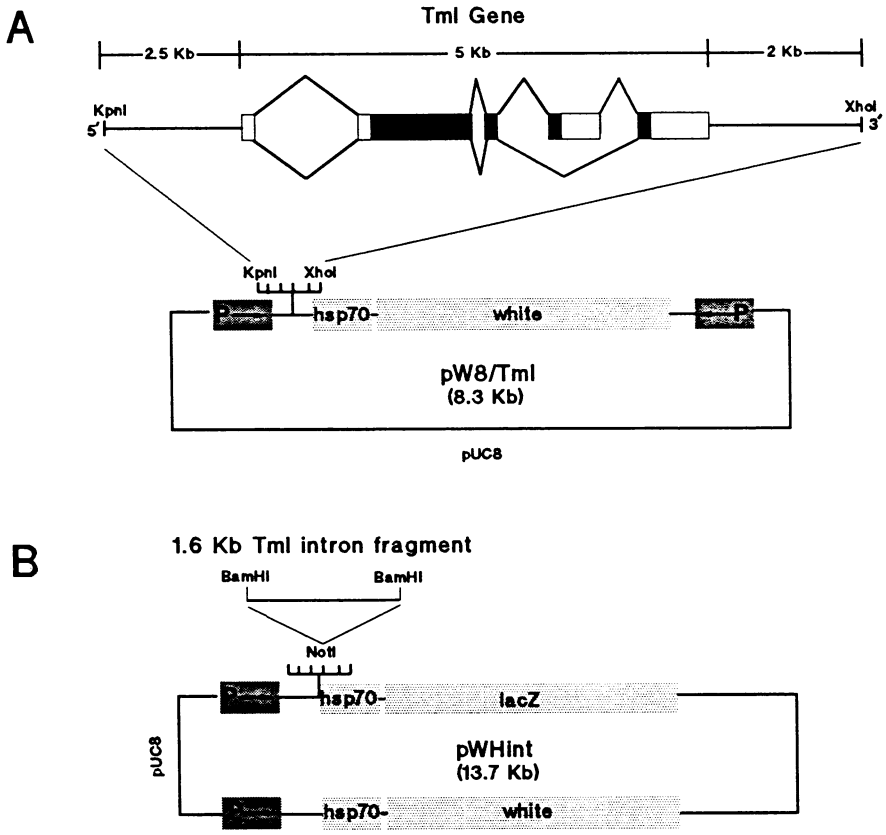


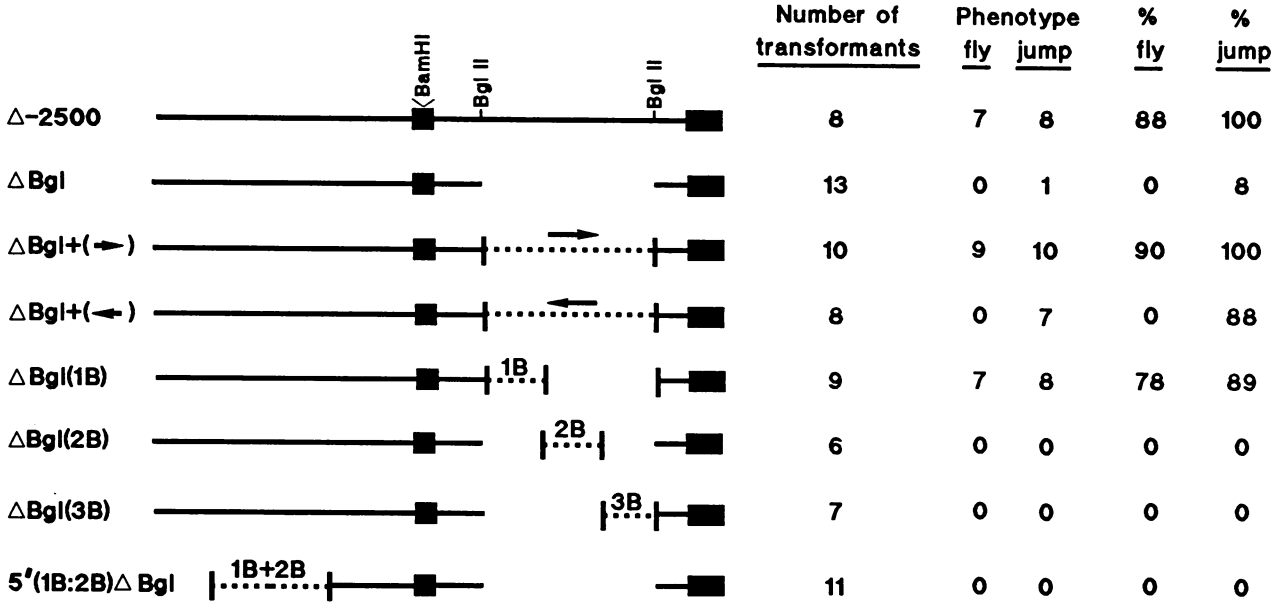
FIG. 1. (A) The pW8/TmI transformation vector. *Drosophila* genomic DNA (9.5 kb) containing the TmI gene and flanking sequences (upper part of diagram) was inserted between the *Kpn*I sites and *Xho*I sites of pW8 (see Materials and Methods). The five exons of the TmI gene are indicated by boxes in which open areas are untranslated regions and solid areas are protein-coding sequence. Transcription of the TmI gene is from left to right. The exon splicing pathways which generate the TmI mRNAs in IFM and TDT muscle are shown on top, and the alternative splicing pathway which produces TmI mRNAs in somatic and visceral muscle of the embryo, larva, and adult is indicated below. In the diagram of pW8, the P-element termini (P), *hsp70* promoter sequence (hsp70), and white gene sequences are shown. (B) The pWHint transformation vector containing the white gene as a selectable marker and the 1.6-kb *Bam*HI fragment inserted into the *Not*I site of the *hsp70-lacZ* gene.

of the TmI gene (30). The consequence of this insertion is that the gene produces greatly reduced levels of a truncated TmI mRNA in IFM and TDT muscle (30, 38). This reduced expression of the IFM-TDT tropomyosin isoform results in the observed flightless and jumpless phenotypes in homozygous *Ifm(3)3* flies. Heterozygous *Ifm(3)3* flies are also flightless, but they can jump. No defects in the production of somatic and visceral muscle TmI RNA or protein have been noted in *Ifm(3)3* flies. In a series of P-element-mediated transformation experiments, we demonstrated that the cloned TmI gene can rescue the mutant phenotypes and restore TmI RNA and protein to near-wild-type levels when introduced into *Ifm(3)3* mutant flies (52). Significantly, it was shown that a minimum dosage of two copies of the inserted TmI gene per cell are necessary to restore flight while one copy of the transgene is sufficient to restore the ability to jump. These results made feasible the identification of *cis*-acting regulatory elements of the TmI gene by introducing in vitro-mutagenized TmI sequences into *Ifm(3)3* flies and analyzing their effects on phenotypic rescue and on mRNA levels. The results presented here have allowed us to identify a muscle-regulatory enhancer element in intron 1 of the gene that can promote expression in adult muscle and rescue of the mutant phenotypes from an heterologous promoter.

MATERIALS AND METHODS

DNA cloning and plasmid preparation. In vitro mutagenesis of the TmI gene was carried out by using the TmI P-element transformation vector as has been described previously (52). This plasmid, p13/TmI (Fig. 1A), contains the TmI gene, with 2.5 kb of 5'-flanking DNA, inserted into the P-element transformation vector pUChsneo of Steller and Pirrotta (50). Initially, a 10-bp *Bam*HI linker was inserted into exon 1 of the TmI gene at the *Ava*I site (+10 bp) by partially digesting p13/TmI with *Ava*I, filling in the ends and attaching the *Bam*HI linker. Similarly, a *Sac*I site 2.5 kb upstream of the TmI transcriptional start site was converted to a *Kpn*I site, resulting in the plasmid Kp13/TmIBL (*Kpn*-p13/TmI/*Bam*HI linker). A *Kpn*I-*Xho*I fragment containing the TmI gene was then transferred from the pUChsneo vector to the pW8 P-element vector (31), which utilizes the white gene as a dominant selectable marker for transformation, to generate the pW8/TmI deletion vectors (Fig. 1A and 2).

The ΔBgl deletion construct, designated Δ*Bgl*, was constructed by deleting the 1,240-bp *Bgl*II fragment (+413 bp to +1653 bp) in intron 1 of the pW8/TmI plasmid. To reinsert the *Bgl*II fragment, the 1,240-bp fragment was isolated from



	Number of transformants	Phenotype fly	Phenotype jump	% fly	% jump
Δ-2500	8	7	8	88	100
ΔBgl	13	0	1	0	8
ΔBgl+(→)	10	9	10	90	100
ΔBgl+(←)	8	0	7	0	88
ΔBgl(1B)	9	7	8	78	89
ΔBgl(2B)	6	0	0	0	0
ΔBgl(3B)	7	0	0	0	0
5'(1B:2B)Δ Bgl	11	0	0	0	0

FIG. 2. Phenotypic analysis of *Ifm(3)3* flies transformed with deletions and rearrangements of intron fragments of the TmI gene. The extent of the remaining 5'-flanking region and exon 1 (with *Bam*HI linker) and part of exon 2 are shown schematically (The deletions are not drawn to scale.) See Materials and Methods for a description of the phenotypic analysis. Exons are in black.

agarose gels and religated to *Bgl*II-digested ΔBgl. Plasmids with both the natural and the inverted orientation of the *Bgl*II fragment were obtained, i.e., Δ*Bgl*+(→) and Δ*Bgl*+(←).

To clone the rearrangements of intron 1, the following manipulations were made. First, the 1,240-bp *Bgl*II fragment was trisected into three subfragments by restriction at the *Eco*RI and *Hin*dIII sites. This generated DNA fragments 355, 535, and 350 bp in size. These fragments (referred to as 1B, 2B, and 3B, respectively) were then filled in with Klenow fragment, and *Bgl*II linkers were attached. After restriction with *Bgl*II the three fragments were isolated and then separately ligated back into the *Bgl*II site in TmIΔBgl, thereby generating ΔBgl(1B), ΔBgl(2B), and ΔBgl(3B). The TmI construct, 5'(1B:2B)ΔBgl, containing the 890-bp 1B and 2B fragment fused to the 5' end of the gene, was cloned by digesting the 1,240-bp *Bgl*II fragment with *Bgl*II and *Eco*RI, filling in the ends, attaching *Eco*RI linkers, and ligating it into the *Eco*RI site 790 bp upstream of the transcription start site of TmIΔ-790ΔBgl.

The heat shock 70 protein gene (*hsp70*) promoter-TmI gene fusion (hspTmI) was made by a two-step cloning scheme. First, TmIΔ-790 was digested with *Kpn*I and *Bam*HI and ligated to a 240-bp *Kpn*I-*Bam*HI fragment containing the *hsp70* gene which was subcloned from the plasmid pHSREM (32). In the second step, the intermediate construct generated from the first step was digested with *Bam*HI and religated to the original *Kpn*I-*Bam*HI-digested TmIΔ-790 to replace the 1.7-kb TmI *Bam*HI fragment to generate hspTmI. To make hspTmIΔBgl, the plasmid TmIΔBgl was first digested with *Kpn*I and *Bam*HI and then ligated to the 240-bp *Kpn*I-*Bam*HI fragment containing the *hsp70* promoter-leader fragment. The *hsp70-lacZ* reporter gene, pWHint, construct (Fig. 2B) was made by first inserting a *Kpn*I-*Pst*I fragment containing the *hsp70* promoter from the pHSREM plasmid (32) into the *Kpn*I site and an artificial *Pst*I site in the P-element vector, pWATG-lacI containing the *Escherichia coli lacZ* gene (33). pWHint was made by blunt-end ligation of a 1.6-kb *Bam*HI TmI gene intron fragment (+12 to +1597) into a *Not*I site upstream of the *hsp70* promoter. All DNA was prepared essentially as described previously (35).

P-element-mediated transformation and the generation of homozygous lines. The *Ifm(3)3* mutation was isolated from ethyl methanesulfonate-treated Canton S males (38). *Ifm(3)3* flies were obtained from Y. Hotta and crossed into a white background to generate homozygous *Ifm(3)3;white* flies, which were the recipients for all transformation deletion experiments. pWHint was transformed into *wt/wt* flies.

P-element transformations were carried out essentially as described previously (49, 52). Eclosed (G_0) flies were backcrossed to the recipient fly stock, and transformed progeny were readily detected by their light-orange to deep-red eye color. The white P-element vectors (Fig. 1) allow for a simple strategy to generate homozygous transformants in only two generations without the aid of balancer chromosomes (31). To generate homozygotes, orange- or light-red-eyed G_1 flies were amplified by backcrossing them to the recipient fly stock and the transformed progeny were subsequently pair mated, leading to darker-orange- or darker-red-eyed homozygous transformants. Southern blot analysis (48) of genomic DNA prepared from each transformed line was used to determine the copy number and to verify the integrity of transgenic inserts. Only homozygous transformants containing a single, unrearranged copy of the transgene per haploid genome were analyzed in this study.

Phenotypes of transformed flies. Flies rescued by P-element transformation with the TmI gene show variations in the rescued phenotype (52). In previous experiments a flight tester was used to quantitate flight and a close correlation was demonstrated between the degree of rescue and transgenic TmI RNA levels. Because of the large number of transformants generated in the experiments presented here, it was impractical to measure flight for all of the transformants by using the flight tester. Accordingly, phenotypic analysis was done by sight. Transformed flies which showed

no rescued phenotype were considered flightless and jumpless. Transformed flies were judged en masse to be capable of either hopping (the lowest level of rescue), jumping (a more elevated hop), or flight. Although subtle differences do exist among lines with similar recorded phenotypes, no attempt was made to categorize these distinctions in this report.

Preparation of RNA and analysis by primer extension. Total RNA was prepared from 1- to 2-day-old whole flies by precipitation from guanidinium isothiocynate and guanidine hydrochloride essentially as described previously (12). Primer extension analysis was carried out essentially as described previously (23) with the modifications described below. Extensions were initiated from an exon 2 23-nucleotide (nt) single-stranded oligonucleotide primer (5′-GACT TACAAGGGAAGAAAACTGG) complementary to the 5′ end of exon 2 (+2163 to +2185). The primer was first 5′ end labeled with ^{32}P by using polynucleotide kinase as described previously (35) and approximately 20,000 cpm of primer were hybridized to 20 to 30 μg of total adult RNA for 3 h at 37°C. Following quick chilling in an ice-ethanol bath, the annealed RNA-DNA hybrid was added to a reaction mixture consisting of 50 mM Tris-HCl (pH 8.4), 80 mM NaCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM each deoxynucleoside triphosphate. The reaction was incubated with 200 U of reverse transcriptase (Bethesda Research Laboratories) for 1 to 3 h to allow for the extension of the annealed primer. The reaction products were recovered by ethanol precipitation and analyzed by electrophoresis into 8% polyacrylamide–8.3 M urea sequencing gels followed by autoradiography of the gel. Quantitative analysis of extension products was determined by directly cutting out the region of the gel containing the desired bands, crushing each sample, and counting the radioactivity in scintillation cocktail. For the endogenous TmI gene extension products the region of the gel encompassing bands corresponding to the extension products at 80, 81, and 83 nt was cut out and quantitated, and for the transgene extension products the region containing the bands at 90, 91, and 93 nt and higher was cut out and counted (see the RNA analysis in the Results section for a description of these bands).

Heat shock schedules. Initially, heat shock of embryos, larvae, pupae, or adults was carried out by incubation at 37°C for 1 h per day. In an attempt to increase the probability that the transgene was being induced during critical stages of myogenesis, pupae were also shocked continuously throughout pupation at 30 to 33°C, but at these extreme temperatures very few adults eclosed. It has been reported that heat shock inhibits splicing of some mRNAs (54). The effects of heat shock on TmI gene expression remain unknown. To avoid this potential inhibition of splicing, a mild heat shock, which induces the synthesis of heat shock proteins, was administered prior to the severe heat shock. This kind of treatment has been shown to protect processing (54). In subsequent experiments, heat treatments were always carried out by preheating pupae at 35°C for 30 min followed by a more severe shock at 37 to 38°C, three times a day for approximately 1 to 3 h. When heat shocking embryos, care was taken to prevent desiccation by heating embryos while they were wrapped in damp paper towels. For RNA extraction from flies less than 1 day old, flies that had been heat shocked as pupae to promote rescue were heat shocked for 1 h, allowed to recover at 23°C for 0, 2, or 5 h, and then stored frozen at −80°C until the time of RNA extractions.

β-Galactosidase staining of whole flies. Adult flies, 1 to 2 days old, were stained for β-galactosidase activity as described previously (24) with slight modifications. Adults were frozen in buffer A (24) containing 1% glutaraldehyde, and the thoraces were either bisected longitudinally down the midline or trimmed along the side with a razor blade to remove the cuticle and expose the underlying muscle. The thawed flies were fixed for 15 min, rinsed in buffer A, and stained for 2 h at 37°C in the dark.

RESULTS

Analysis of the phenotypes of transformed lines. We have used both biological and biochemical assays to analyze the expression of TmI deletion mutations in transformed flies. First, to determine the physiological effect of the deletion mutations on IFM and TDT muscle function and as a rough measure of TmI gene expression, we assayed the ability of the deletion constructs to rescue the *Ifm(3)3* mutant phenotypes. Homozygous *Ifm(3)3* flies can neither fly nor jump, whereas *Ifm(3)3* flies heterozygous for the endogenous TmI gene or a P-element-transformed TmI gene can jump but cannot fly (52). *Ifm(3)3* flies homozygous for the TmI transgene can fly and jump (52). Thus, a single gene dose is sufficient for proper TDT muscle assembly and normal function but two doses are required for IFM assembly and function. A complete description of the effects of tropomyosin dosage on muscle assembly and function will be presented elsewhere (45a). To define *cis* regions required for TmI expression, we constructed a series of 5′ (not shown) and intron deletions. We were particularly interested in the intron region since both tropomyosin genes and several other *Drosophila* genes have small first exons encoding mRNA leader sequences, followed by relatively large first introns. Figure 1A shows the entire structure of the TmI gene and the P-element transformation vector used for most of the deletions. Figure 2 shows the results of phenotypic analysis of the intron deletions tested.

The TmI gene containing 2,500 bp of 5′-flanking DNA shows rescue of flight in seven of eight transformed lines and jumping in all eight transformed lines (Fig. 2). Additional 5′ deletions to −1900 and −790 bp yield 100% rescue of both phenotypes in 10 transformed lines (not shown). The intron 1 deletion shown (ΔBgl) is a 1,240-bp deletion of DNA contained within two *Bgl*II sites (Fig. 2). This deletion resulted in the total reduction of rescue to 0%; i.e., none of the 13 transformed lines was capable of flight (only 1 of 13 of these lines jump). To verify that this deletion was indeed the cause of such a drastic reduction in rescue and not a cloning artifact, we cloned the *Bgl*II fragment back into the *Bgl*II site of the deletion plasmid in the same orientation. This construct, ΔBgl+(→), restored flight and jumping in 9 of 10 independently transformed lines, demonstrating that the result with the 1,240-bp deletion was genuine and not a cloning artifact. Enhancer elements are generally capable of functioning in an orientation-independent manner. To test the possibility that the *Bgl*II fragment had enhancerlike activity, we cloned the 1,240-bp fragment in the reverse orientation. This clone, ΔBgl+(←), did not restore flight (in eight of eight lines) but restored jumping in seven of eight homozygous lines. This indicates that sufficient TmI gene activity is retained with the intron sequences in the reverse orientation to restore jumping.

In an attempt to define the boundaries of the region responsible for the results seen with the Δ*Bgl* lines, the 1,240-bp fragment was trisected into three smaller subfragments (see Materials and Methods). Fragments of 355 bp (1B), 535 bp (2B), and 350 bp (3B) were separately cloned

back into the *Bgl*II site to generate the TmI deletion plasmids ΔBgl(1B), ΔBgl(2B), and ΔBgl(3B). Only ΔBgl(1B) was capable of restoring either flight or jumping (Fig. 2). Of nine transformed lines containing this 355-bp fragment, seven showed restored flight and eight showed restored jumping. When the other two plasmids, ΔBgl(2B) and ΔBgl(3B), were transformed into *Ifm(3)3*;*white* flies, there was no rescue of flight or jumping (zero of six and zero of seven lines, respectively). Since enhancer elements are also capable of functioning in a position-independent manner, it was important to determine whether the 1B fragment, which restored flight and jumping by itself when reinserted into the intron, could function if placed upstream of the transcriptional start site. To test this, the 1B and 2B fragments together were cloned 790 bp upstream of a TmI gene containing a deleted *Bgl*II fragment (−790 bp is the smallest amount of 5′ DNA tested that still gives 100% rescue of both mutant phenotypes). This 5′ 1B:2BΔBgl construct showed no rescue in 11 transformed lines.

RNA analysis. We have performed quantitative analysis of RNA levels on 1- to 2-day-old adult transgenic flies by primer extension. A 10-bp *Bam*HI linker was inserted into exon 1 of all deletion constructs so that we would be able to differentiate endogenous (80-nt extensions) TmI expression from that of the transgene (90-nt extensions) and determine the relative levels of transcription among the various deletion mutants. As shown previously (4), there exist three clustered sites of transcriptional initiation from the endogenous TmI gene (Fig. 3, E). The transgenic RNA mimics this three-site cluster precisely 10 bp above the endogenous bands (Fig. 3, T). However, in addition to these expected start sites, there is a series of additional larger minor bands. These additional bands were never observed with RNA extracted from either nontransformed lines [*white*;*Ifm(3)3* flies] or lines transformed with a TmI gene without the linker. It is possible that because of the linker insertion, proper positioning of RNA polymerase II at the normal start site was sometimes disrupted, leading to the observed extraneous sites of initiation.

As a first step in our analysis, we analyzed a group of transformants (a 5′ deletion) that displayed the widest range of phenotypes to quantitatively determine position effect variation (41) and to determine the extent to which transgenic TmI RNA expression correlated with the observed phenotypes. These results (45a) demonstrated that the relative levels of transgenic TmI mRNA correlate with the observed phenotype. Because of the close ratios obtained between transformed lines with similar phenotypes, it was decided to analyze pools of flies of like phenotype (50 males and 50 females from each line) instead of analyzing each of the independently transformed lines separately.

RNA analysis of the intron deletions is shown in Fig. 3 and Table 1. In flies containing the 1,240-bp ΔBgl deletion, which completely reduced the ability of the transgene to restore flight or jumping in 12 of 13 transformed lines, RNA expression was reduced to a normalized value of 9% of the endogenous TmI gene. When the 1,240-bp intron fragment was reinserted into its proper orientation, TmI gene expression was restored to approximately 95% of the normalized levels. Interestingly, expression ratios from the transgene with the *Bgl*II fragment in the inverted orientation averaged 0.61 ± 0.03, or about 77% of what was observed for the highest-expressing transformed lines. Seven of eight of these transformed lines with the *Bgl*II fragment inverted showed restoration of jumping. These data, together, confirm that a large amount of activity can be restored when the intron sequences are in the reverse orientation.

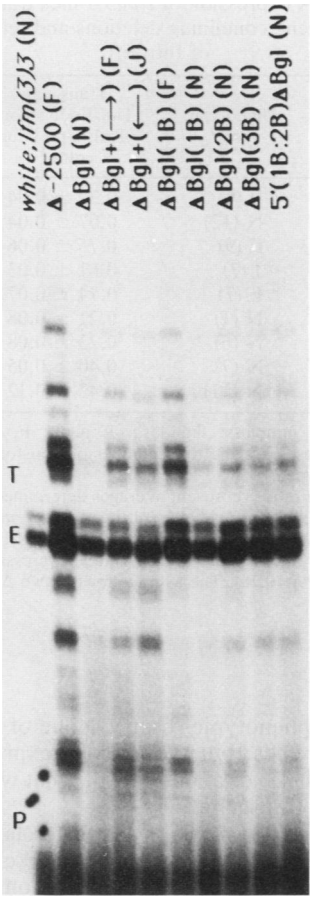


FIG. 3. Primer extension analysis of RNA from flies transformed with internal deletions and rearrangements of intron 1 of the TmI gene. The transgene has been marked with a 10-bp *Bam*HI linker insertion and produces extended products 10 nt larger then the endogenous gene. The lanes correspond to the transformants indicated in Table 1, and the letters N (jumpless, flightless), J (jump), and F (fly) indicate the observed phenotypes. The regions corresponding to the TmI primer (P), endogenous (E), and transgenic (T) mRNA are indicated.

RNA expression in the ΔBgl(1B) transgene lines is very high. The average expression ratio from all of these transformants is 0.63, which is 80% of the mRNA levels determined for the Δ-2500 TmI transgene. ΔBgl(1B) was the only one of the three intron constructs capable of restoring jumping (eight of nine) and flight (seven of nine). ΔBgl(1B)N, which showed no phenotypic rescue and had an expression ratio of 0.21, contains a TmI transgene that has probably integrated into an inactive chromosomal region. If the expression ratio of the seven flying lines is averaged without this lower-expressing line, the normalized ratio would be 95% of the mRNA levels determined for the Δ-2500 TmI transgene. The ΔBgl(2B) and ΔBgl(3B) constructs showed no restoration of flight or jumping. Surprisingly, ΔBgl(2B) and ΔBgl(3B) also express the transgene at significant levels. Expression ratios for these clones averaged 0.23 for ΔBgl(2B) and 0.40 for ΔBgl(3B), or normalized values of 29 and 51%, respectively. In addition, 5′(1B:2B)ΔBgl expressed the transgene at significantly higher levels than we expected

TABLE 1. RNA expression of *Ifm(3)3* flies transformed with a cloned TmI gene containing deletions and rearrangements of Intron 1

Transgene	Phenotype (no.)[a]	Transgenic TmI/Endogenous TmI expression ratio[b]	Normalized ratio[c]
Δ-2500	F (6)	0.79 ± 0.04	1.00
ΔBgl	N (12)	0.07 ± 0.04	0.09
ΔBgl+(→)	F (9)	0.75 ± 0.06	0.95
ΔBgl+(←)	J (7)	0.61 ± 0.03	0.77
ΔBgl(1B)	F (7)	0.74 ± 0.07	0.94
ΔBgl(1B)	N (1)	0.21 ± 0.08	0.27
ΔBgl(2B)	N (6)	0.23 ± 0.08	0.29
ΔBgl(3B)	N (7)	0.40 ± 0.05	0.51
5′(1B:2B)ΔBgl	N (11)	0.42 ± 0.12	0.54

[a] Phenotypes: N, jumpless, flightless; J, jump; F, fly and jump. The numbers in parentheses are the total number of transformed lines analyzed sharing that phenotype.

[b] RNA expression ratios represent averages determined from two separate primer extension gels ± standard error of the mean. A weighted average was calculated for the two indicated phenotypes from ΔBgl(1B) and is 0.63.

[c] These ratios were normalized to the average expression ratio determined from Δ-2500 and Δ-790 (0.78). The average for the two ΔBgl(1B) phenotypes is 0.80.

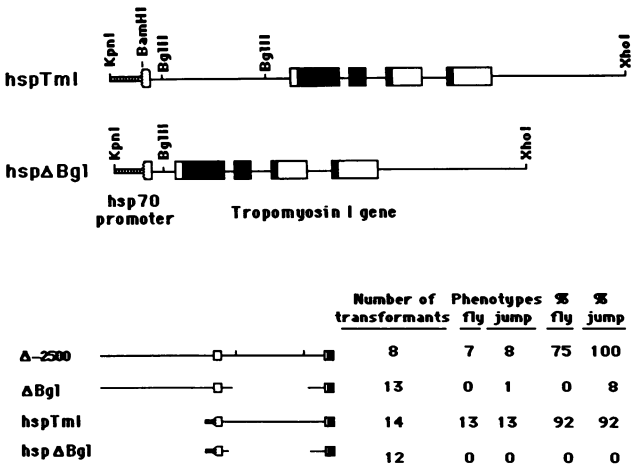


FIG. 4. Transformation of TmI flightless *Ifm(3)3* mutant flies with *hsp70*-TmI gene fusions. At the top of the figure schematic diagrams of the hspTmI and hspΔBgl gene fusions are shown. The lower part of the figure describes the phenotypes of the transformed lines. The Δ-2500 and ΔBgl clones are shown as references.

on the basis of phenotypic rescue. None of the 11 transformants jumps or flies, but the average expression ratio for these lines was 0.42, or about 54% of what was observed for the Δ-2500 TmI transgene.

Analysis of flies transformed with heterologous *hsp70* promoter-TmI gene fusions. Since enhancerlike elements function by increasing the rate of transcription and are sometimes capable of functioning with a heterologous promoter (36, 45), it was of interest to determine whether the *Bgl*II fragment could drive transcription from a heterologous nonmuscle promoter. In addition, it was important to verify that deletion of the *Bgl*II fragment was affecting transcriptional initiation and not some posttranscriptional process. If the intron *Bgl*II deletion was acting at the level of transcription, we would expect the rate of transcription to be reduced in the ΔBgl lines compared with that in TmI gene constructs containing the *Bgl*II fragment. If, on the other hand, rates of transcription were approximately the same, we might expect some posttranscriptional process such as mRNA splicing and/or stability to be affected by the deletion. Intron sequences have been implicated in posttranscriptional processes (10, 13, 22, 42, 55). Unfortunately, differentiating transcriptional control of transgenic and endogenous TmI heterogeneous nuclear RNA (hnRNA), in the context of a nuclear runoff experiment (21), is not trivial, and therefore this problem was investigated further by a different approach. Two TmI gene constructs, with and without the *Bgl*II fragment, were ligated at exon 1 of TmI to the exon 1 and promoter region of the *Drosophila hsp70* gene, resulting in the fusion gene plasmids hspTmI and hspΔBgl (Fig. 4). The rationale was that, if the intron regulatory element was capable of driving expression of a heterologous promoter and if it was affecting transcription at the level of initiation, it should be possible to overcome this control with the inducible *hsp70* promoter. Subsequently, upon heat shock, it should be possible to observe and quantitate RNA expression from flies containing the hspΔBgl plasmid. If, on the other hand, the intron element was required for faithful processing (stability, splicing, or transport), heat-induced hspΔBgl hnRNA would not be processed and we would not be able to detect mature transgenic mRNA from this chimeric gene construct.

The results of transformations of *Ifm(3)3* mutants with the hspTmI fusion genes are shown in Fig. 4. In 92% of the hspTmI transformants (13 of 14) there was restoration of both jumping and flight, whereas none of the 12 independently transformed lines containing the *Bgl*II deletion showed phenotypic rescue. This result indicates that the intron regulatory region can drive transcription of the *hsp70* promoter. The phenotypes described for the hspTmI and hspΔBgl transformants are those seen when flies were reared at normal temperatures (22 to 25°C). It was of interest to investigate the effect of inducing the hspΔBgl and hspTmI transgenes. Since the adult musculature of the fly is synthesized and assembled during the first 40 to 60 h of pupation (14), we presumed that induction during this period would be optimum for an attempted heat shock induced rescue of the hspΔBgl-transformed *Ifm(3)3* flies. However, it was also possible that induction prior to pupation could influence the establishment of organized and competent myofibers. For these reasons of uncertainty, transformed lines containing the hspΔBgl plasmid were heat shocked under a variety of different schedules and temperatures to determine whether restoration of jumping or flight could be induced.

The most successful rescue was obtained when embryos were allowed to develop at 23°C until pupariation and then heat shocked three times a day (with pre-heat treatment) for 1 to 3 hours at 37 to 38°C throughout pupation. Under these conditions of heat treatment, hopping, jumping, and, on a few occasions, flying (very brief and short flights) were observed. Heat shocking adults only (1 to 2 days old) at 37 to 38°C for 1 to 3 h a day never produced any observable rescue. These results suggest that induction of the TmI gene could occur, even when the internal control element was deleted, and that in a small number of flies, TmI mRNA levels were sufficient to restore hopping and jumping and occasionally flying. The fact that complete rescue was not achieved with heat shock is not surprising and is probably

TABLE 2. Expression ratios of *Ifm(3)3* flies transformed with heterologous *hsp70*-TmI gene fusions

Transgene	Transgenic TmI/Endogenous TmI expression ratio[a]	Normalized ratio	Phenotype[c]
hspΔBgl	0.04 ± 0.01	0.05	N
hspΔBgl[37°C]T_0[d]	0.33 ± 0.02	0.43	H
hspΔBgl[37°C]T_2	0.21 ± 0.01	0.28	H
hspΔBgl[37°C]T_5	0.29 ± 0.06	0.38	H
hspTmI	0.84 ± 0.05	1.10	F
hspTmI[37°C]T_0	1.37 ± 0.07	1.80	F
hspTmI[37°C]T_2	0.98 ± 0.13	1.29	F
hspTmI[37°C]T_5	0.99 ± 0.20	1.30	F

[a] Ratios represent the average calculated from two separate determinations ± standard error of the mean.
[b] Normalized to Δ-2500 (0.79).
[c] Phenotypes: N, jumpless, flightless; H, hop; F, fly (also capable of jumping).
[d] [37°C]T_n indicates that flies (less than 1 day old) were heat shocked at 37°C for 1 h and allowed to recover at 22°C for n (n = 0, 2, or 5) h, and RNA was extracted.

due to either insufficient amounts of mRNA and protein produced during the heat shock or ill-timed heat induction for proper myofiber assembly.

Flies transformed with the hspTmI plasmid expressed the transgene (Table 2) at 110% of the levels observed for the Δ-2500 transgene (100%). This is consistent with the rescue seen in 13 of the 14 lines. The hspΔBgl plasmid, on the other hand, showed approximately 5% of the mRNA levels of Δ-2500 or basal-level promoter function and is consistent with the lack of phenotypic rescue in all 12 transformed lines studied. We next determined whether flies transformed with the two *hsp*-TmI heterologous plasmids could be induced to express stable TmI mRNA. Pupae were heat shocked to promote rescue as above and then heat shocked for 1 h (T_0) as young adults (less than 1 day old). Subsequently, RNA was extracted and subjected to primer extension analysis. Both hspTmI and hspΔBgl, when heat shocked, showed significant induction of TmI mRNA (hspΔBgl[37°C]T_0 and hspTmI[37°C]T_0 in Table 2). Levels of mRNA increased by a factor of about 8 for hspΔBgl, whereas heat-shocked hspTmI expression increased by a factor of approximately 1.6. As a rough means of determining the relative stabilities of these induced TmI mRNAs, flies were heat shocked for 1 h to induce transcription and allowed to recover at 22°C for 0, 2, and 5 hours, and then RNA was extracted. Results from this analysis are also shown in Table 2. Initially, during the first hour of recovery from heat shock, steady-state levels of TmI mRNA from hspTmI and hspΔBgl dropped by about 30 and 36%, respectively. The relative levels, however, did not change significantly between 2 and 5 h after induction. Regardless of the cause of the initial decrease in steady-state levels of TmI RNA, which could be a consequence of the heat shock and/or reflect either mRNA turnover or utilization by the translational apparatus, these experiments demonstrate that, upon heat shock, the hspΔBgl fusion was induced and sufficient amounts of stable mRNA were produced to restore hopping, jumping, and, on a few occasions, flying. Thus the deletion of intron sequences does not result in a difference in the relative stability of the hspΔBgl and hspTmI mRNAs.

Expression of an *hsp70-lacZ* reporter gene under the control of the intron enhancer region is muscle specific. The previous experiments demonstrated that the intron enhancer region could direct expression from the heterologous *hsp70* promoter. They do not address directly whether the muscle control region is sufficient for muscle expression and whether expression is muscle specific. Accordingly, an intron fragment from the TmI gene extending from the synthetic *Bam*HI site at +10 to the *Bam*HI site at +1596 was inserted upstream of the *hsp70-lacZ* reporter gene (Fig. 1B). The region from the *Bam*HI site at position +10 to the *Bgl*II site at position +413 was included to ensure that sequences upstream of the 1B fragment, which might also contain enhancer activity (see Discussion), were included. With this reporter gene construct, the tissue distribution of enhancer activity can be visualized directly by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of the transformed adult flies. Three independently transformed lines were analyzed in whole-mount assays. Figure 5A is a longitudinal cut bisecting the thorax showing X-Gal staining of all of the dorsal lateral IFM. Figure 5B shows a vertical cut along the side of the thorax exposing the stained dorsal-ventral IFM and the TDT muscle extending dorsoventrally and inserting on the mesothoracic leg. Figure 5C is a section of the abdomen showing variable staining of visceral gut muscle and dorsal and ventral somatic muscle of the abdomen. It should be noted that in any given fly, only a fraction of the entire musculature stains positive for β-galactosidase because of the impermeability of the cuticle surrounding the muscle. However, an analysis of several flies from all three transformed lines showed that in each line all identifiable muscle classes stained for β-galactosidase. No staining was observed in nonmuscle structures of transformants, except for occasional staining of the pericardial cells along the dorsal abdominal wall and of the crop, which also stain occasionally in nontransformed white control flies (not shown).

DISCUSSION

The intron muscle regulatory region. P-element-mediated germ line transformation and rescue of the TmI flightless and jumpless *Ifm(3)3* mutant flies have allowed us to study the regulation of expression of the TmI gene in adult flies. In this study we have identified a *cis*-acting region within intron 1 of the gene that is both necessary and sufficient to promote muscle-specific expression in the adult fly. The intron muscle-regulatory region has three properties characteristic of a muscle-specific regulatory enhancer element. First, it has the ability to function at least partially in an orientation- and position-independent manner. The partial expression observed (77% in the inverse orientation and 54% when placed upstream, relative to the unrearranged gene) indicates that some promoter-intron interaction can still occur, but that the spatial relationship of the components of the transcriptional complex formed cannot promote full expression. In this regard it is possible that additional *cis*-acting intron sequences upstream of the *Bgl*II fragment are required for full transcriptional activity. It was for this reason that we included sequences from +10 to +413 along with the *Bgl*II fragment in the *hsp70-lacZ* reporter gene. More recent experiments indicate, however, that these additional sequences are not required (25a).

A second property of the intron fragment is its ability to function with a heterologous promoter. In the experiments described above it was shown that the muscle-regulatory region is able to drive full transcriptional activity of an *hsp70* promoter-TmI fusion gene and restore both flight and jump-

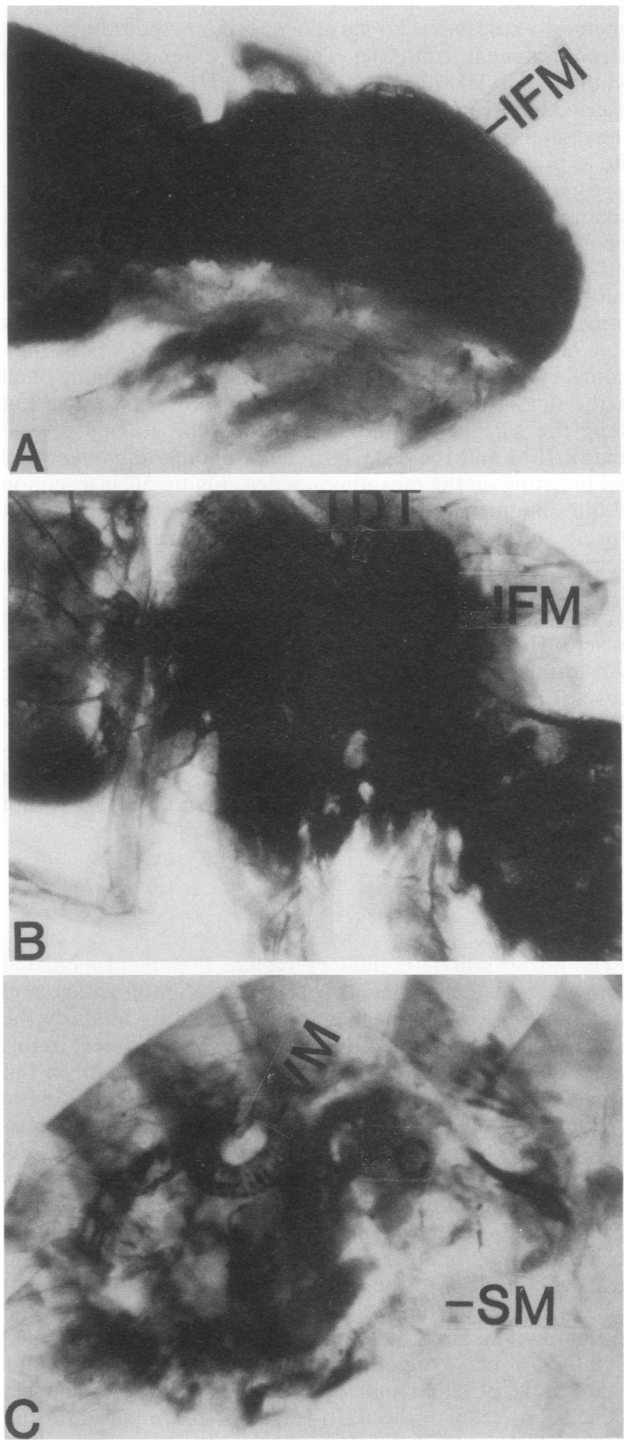


FIG. 5. (A) Whole mount of the thoracic region of an adult fly bisected longitudinally to expose the dorsal median IFM and stained for β-galactosidase activity. (B) Whole mount of the thoracic region of an adult fly. The cuticle on one side of the fly was trimmed to expose the dorsal-ventral IFM and the tergal depressor of the TDT or jump muscle. (C) Whole mount of an adult showing the abdominal region and staining of visceral gut muscle (VM) and somatic muscle (SM) of the abdomen and nonspecific staining of the crop (C).

ing to *Ifm(3)3* flies. Indeed, expression went from a basal expression level measured to be about 5% to approximately 110% of the *Ifm(3)3* TmI gene control, indicating that the muscle-regulatory region not only is capable of driving expression from the *hsp70* promoter but also tightly controls the level of promoter function. These results suggest that although the TmI promoter is a functional promoter, it is not required for muscle-specific expression or for regulating expression at a wild-type level. It is still possible that the TmI promoter has duplicative regulatory functions or functions associated with expression not measured in our assays. However, in our analysis of the TmI 5′-flanking promoter region, we have no evidence suggesting that this region confers developmental or tissue specificity to expression of the gene.

The TmI gene lacks a TATA box homology at the −20 to −30 position, where it is usually found in the majority of eukaryotic genes (25). It is interesting that the *hsp70* promoter contains a functional TATA box located at position −33 to −26, whereas the TmI gene does not have an upstream TATA element. The first TATA sequence in the TmI gene is found at a position 158 bp upstream of the mRNA cap site. This particular TATA sequence is apparently dispensable for low levels of accurately initiated TmI mRNA, since a deletion of 5′-flanking DNA to −146 still retains significant transcriptional activity (45a). There is, however, another TATA sequence downstream of the transcription start site of the TmI gene at position +28 of exon 1. The promoter of the simian virus 40 late genes and the promoter of the mammalian terminal deoxynucleotidyltransferase gene utilize an element overlying the start site to fix the place of initiation (1, 47). Therefore, it is conceivable that the downstream TATA element in the TmI gene could function as the positioning element. It is interesting in this regard that the insertion of the *Bam*HI linker at position +10 results in additional transcriptional initiation sites. If the downstream TATA element in the TmI gene is utilized as a positioning element, it is possible that the insertion of an additional 10 bp into this region affects the normal positioning of RNA polymerase II. This, therefore, could explain the additional transcriptional start sites observed from the TmI transgene inserts. Since the *hsp70*-TmI fusion gene is initiated at the proper (*hsp70*) start site, the TmI muscle enhancer is apparently capable of functioning in conjunction with this heterologous upstream TATA box and with its cognate binding proteins.

Many genes have been shown to contain intron sequences that contribute to the stability or processing of their mRNAs (10, 13, 22, 29, 42, 44, 55). The fact that the hspΔBgl fusion was induced about eightfold over uninduced levels and significant amounts of stable mRNA were produced and present up to 5 h after heat induction indicates that the intron regulatory element is not required for stabilizing TmI hnRNA, but, instead, enhances expression of the TmI gene at the level of transcriptional initiation. The fact that complete rescue was not achieved with heat shock is not surprising and is probably due to either insufficient amounts of mRNA and protein produced during the heat shock or ill-timed heat induction for proper myofiber assembly. Indeed, deletions of the 5′-flanking region that only marginally lower TmI expression can significantly affect phenotypic rescue such that a threshold level of TmI is necessary to restore muscle fiber assembly and function (45a).

A third property of the intron enhancer is that it is capable of muscle-specific transcriptional enhancement of an *hsp70-lacZ* reporter gene in both IFM and TDT muscle and all

other identifiable muscle of the adult. We have extended this analysis to show that expression in these transformants is also muscle specific for all embryonic and larvae muscle (25a). This tissue-specific enhancement is position independent as well as promoter independent. Thus, *cis*-acting elements required for expression in all muscle appear to be contained with the intron region. This is in contrast to the situation for β_3-tubulin gene, the only other *Drosophila* muscle gene for which information is available, for which visceral muscle expression in the embryo is controlled by intron sequences while expression in other muscle of the embryo and larva is controlled by 5′ upstream sequences (20).

IFM-TDT enhancer. Since phenotypic rescue of IFM and TDT muscle function is quantitative in the mutant (52; see above), we have been able to correlate TmI expression levels with muscle function. This has allowed us to define a 350-bp *cis*-acting DNA region within a 1,240-bp *Bgl*II fragment of intron 1 of the gene that is required for complete rescue of the IFM and TDT mutant phenotypes. This region, contained within the 1B fragment located between positions +413 and +763, is able to stimulate an average increase in RNA expression to near wild-type levels as determined by measuring mRNA levels. These high levels of RNA expression correlate with the rescue of flightlessness and thus localize the *cis*-acting element required for IFM and TDT muscle expression to this region.

The intron region is likely to be complex, however, and can be subdivided further on the basis of primer extension analysis of TmI mRNA levels. A second domain of *cis*-acting transcriptional activity is contained within the 2B and 3B regions. The 2B fragment was able to confer an average threefold increase, while the 3B domain showed a sixfold increase, in TmI RNA expression in adults when compared with the levels of expression determined for the *ΔBgl* lines. This result was surprising in light of phenotypic data which suggested that fragment 1B alone was capable of restoring jumping and flight and that regions 2B and 3B were not required for expression in adult IFM and TDT muscle; it raises the question of the role of the 2B-3B region in controlling TmI gene expression. One possibility is that additional IFM-TDT elements span the 2B-3B region but do not promote high enough levels of TmI mRNA for rescue. A second possibility is that intron sequences within the 2B-3B regions are involved in regulating TmI expression in non-IFM-TDT muscle. Indirect support for this notion comes from the finding that the *Drosophila* β_3-tubulin gene is regulated by separate embryonic somatic and visceral muscle *cis*-acting elements located 5′ of the gene and within intron 1, respectively (20). A third possibility is that the 2B-3B region contains enhancer elements that regulate temporal or relative levels of expression (presumably in non-IFM-TDT muscle). These questions will be resolved with the aid of TmI-*lacZ* reporter gene constructs containing the 1B, 2B, and 3B fragments.

A model for the involvement of *cis*-acting elements in TmI gene expression. On the basis of the results presented here and results published for other genes that are regulated by internal *cis*-acting sequences (7, 51, 56), a hypothetical model can be proposed to account for the activation and regulation of TmI gene transcription. Expression of the TmI gene is controlled by a minimal promoter located 5′ to the gene and a muscle-specific enhancer(s) within intron 1. The interactions which occur between the intron muscle element(s) and 5′-flanking promoter sequences are mediated through the presence of sequence- and tissue-specific binding proteins. If multiple muscle-specific regulatory elements are indeed found to reside within intron 1 of the gene (i.e., IFM and/or TDT specific and non-IFM/TDT specific), we can postulate that different sets or combinations of muscle-specific factors could direct differential TmI gene expression. The interactions between the 5′ promoter and intron control elements could be stabilized by looping out the intervening DNA (8, 28) which may in turn bring intron regulatory factors that are associated with one of the two intron regions in close proximity to the transcriptional initiation complex. It is possible that different factors favor separate, unique promoter-enhancer interactions that in turn dictate the muscle type specificity of TmI gene expression.

A prominent structural feature of the TmI gene and the evolutionarily related tropomyosin II (TmII) gene is the presence of a relatively large intron 1 separating an exon 1 encoding an approximately 50-bp leader sequence from a second leader/protein-coding sequence second exon (26). It should be noted that the remaining 14 introns of the TmII gene have been deleted or rearranged in the TmI gene. Furthermore, a similar structural arrangement of small exon 1–intron-exon 2 has been noted for several other *Drosophila* and vertebrate muscle and nonmuscle genes and suggests a possible mechanism for evolution of transcriptional regulatory control elements through recombination and rearrangement. We have determined that the TmII gene also contains a muscle-regulatory region within intron 1 with properties similar to the TmI gene (37a), and, as mentioned above, the β_3-tubulin gene also has a large intron 1 containing sequences required for muscle expression (20). Such an arrangement may form the basis for coordinate muscle gene control in which *cis*-acting regions are recombined to provide a common mechanism for regulating muscle genes in *D. melanogaster*.

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