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Transcription of *Drosophila* Troponin I Gene Is Regulated by Two Conserved, Functionally Identical, Synergistic Elements[D]

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The Drosophila *wings-up A* gene encodes Troponin I. Two regions, located upstream of the transcription initiation site (upstream regulatory element) and in the first intron (intron regulatory element), regulate gene expression in specific developmental and muscle type domains. Based on *LacZ* reporter expression in transgenic lines, upstream regulatory element and intron regulatory element yield identical expression patterns. Both elements are required for full expression levels in vivo as indicated by quantitative reverse transcription-polymerase chain reaction assays. Three myocyte enhancer factor-2 binding sites have been functionally characterized in each regulatory element. Using exon specific probes, we show that transvection is based on transcriptional changes in the homologous chromosome and that Zeste and Suppressor of Zeste 3 gene products act as repressors for *wings-up A*. Critical regions for transvection and for Zeste effects are defined near the transcription initiation site. After in silico analysis in insects (*Anopheles* and *Drosophila pseudoobscura*) and vertebrates (*Ratus* and *Coturnix*), the regulatory organization of *Drosophila* seems to be conserved. Troponin I (TnI) is expressed before muscle progenitors begin to fuse, and sarcomere morphogenesis is affected by TnI depletion as Z discs fail to form, revealing a novel developmental role for the protein or its transcripts. Also, abnormal stoichiometry among TnI isoforms, rather than their absolute levels, seems to cause the functional muscle defects.

INTRODUCTION

Contractile protein systems are widely represented in most cell types as a force generator device (Davison *et al*., 2000). In muscles, troponin I (TnI) is a key element in the protein complex that regulates sliding of thin over thick filaments (Farah and Reinach, 1995; Geeves and Lehrer, 1998; Squire and Morris, 1998; Maytum *et al*., 2003). Several TnI protein isoforms are generated, either from transcription of independent genes (e.g., vertebrates) or from differential splicing of a single gene primary transcript (e.g., *Drosophila*). Amino acid substitutions in constitutive or alternatively spliced exons in TnI can lead to pathological conditions such as familial hypertrophic cardiomyopathy (Carrier *et al*., 1993; Coonar and McKenna, 1997) and distal arthrogryposis (Sung *et al*., 2003), due to abnormal interactions with other sarcomere components. Also, TnI is a relevant indicator of heart failure (Lewinter and Vanburen, 2002) and a potent angiogenesis inhibitor through its interaction with polycystin-2 (Li *et al*., 2003). The potential applications that this knowledge could provide, however, are handicapped by the scant information on the regulatory mechanisms of TnI gene expression. This issue is particularly relevant in the context of future gene therapy strategies and justifies this in vivo study of the regulatory mechanism of the *Drosophila* homologue. In addition, this study takes advantage of the fact that TnI in *Drosophila* is encoded by a single gene, *wings up A* (*wupA*), and that isoform replacement during normal development as in the mouse heart (Siedner *et al*., 2003) does not take place. These features render the task amenable.

Transcription regulation is an elaborated process that requires specific interactions between genomic sequences and components of the transcriptional machinery resulting in local structural changes of the chromatin (Davidson *et al*., 2002). In addition to these *cis*-effects, transcriptional changes can be elicited in *trans*- on the homologous gene copy through a largely unknown mechanism thought to depend on correct chromosomal pairing at the gene locus (but see Goldsborough and Kornberg, 1996). In vertebrates, three different genes (fast, slow, and cardiac), encode specialized forms of TnI, whereas in *Drosophila*, the single gene *wings-up A* encodes 10 TnI protein isoforms (Barbas *et al*., 1991). The gene is expressed shortly after the monolayer of mesodermal cells is determined by the sequential expression of *twist*, *Dmef2*, *tinman*, and other transcription factor-encoding genes (stages 6–7 or 3–4 h of development) (Thisse *et al*., 1987; Azpiazu and Frasch, 1993; Bour *et al*., 1995; Lilly *et al*., 1995; Taylor, 2000). Muscle progenitor cells are determined immediately afterwards, and their descendants, the muscle founder cells, become recognizable by the end of stage 11 (7–8 h) (Carmena *et al*., 1995; Ruiz and Bate, 1997). Cell fusions between founder and adjacent fusion-competent cells begin at stage 13 (9–10 h) leading to muscle syncitia (Baylies *et al*., 1998; Paululat *et al*., 1999; Taylor, 2002). The earliest TnI transcripts are detected at 4 h by reverse transcription-polymerase chain reaction (RT-PCR) and are maintained thereafter as indicated by in situ hybridization (Prado *et al*., 1999) (Figure 1A). By stage 13, high levels of TnI transcripts can be observed in segmental arrays of muscle primordia of fused cells when they initiate differentiation

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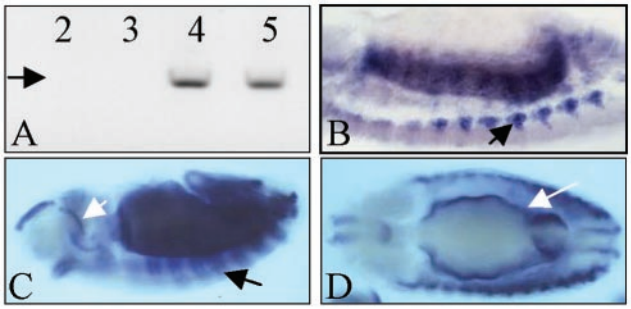

Figure 1. wings-up A expression in the embryo. (A) RT-PCR of 2- to 5-h embryo extracts primed for TnI. Expression of a 1.2-kb cDNA (arrow) can be seen from stage 7 (4 h) onwards. Numbers indicate hours of development from 30 min. Egg-laying periods of the CS strain. (B) Lateral view of a 10-μm section of a stage 12 embryo revealing TnI transcripts in somatic muscle primordia (arrow) by in situ hybridization. (C) Lateral view of a whole mount stage 12 embryo showing TnI transcripts in segment arranged somatic developing muscles (black arrow), and in the foregut (white arrow). (D) Dorsal view of a whole mount stage 14 embryo showing TnI expression in developing visceral muscles (white arrow).

and stretch toward anchoring apodemes (Figure 1B). By stage 14, TnI transcripts are prominent in visceral and somatic developing muscles (Figure 1, C and D). Thus, although TnI is usually considered a muscle differentiation marker involved in the regulation of sarcomere contraction, it is clearly expressed well before muscle founder cells begin to fuse.

After this introductory description of the early TnI gene expression, we address the regulatory mechanisms for *wings-up A* transcription, characterize some of the transcription factor binding sites, and measure in vivo the transcriptional *cis*- and *trans*-effects produced by rearrangements of the regulatory regions. The regulatory design described here seems conserved in other *Drosophila* muscle genes and, most relevant, in TnI encoding vertebrate genes. Furthermore, we demonstrate that TnI is required for muscle morphogenesis, as opposed to muscle contraction only, and that the unbalance of TnI isoforms results in severe muscle dysfunction.

MATERIALS AND METHODS

Fly Strains and Crosses

Df(1)23437 and the point mutations hdp^2 and hdp^3 were described previously (Beall and Fyrberg, 1991; Barbas *et al*., 1993; Prado *et al*., 1995, 1999). Regulatory mutants *PL87* and *PG31* were provided by H.M. Bourbon (Bourbon *et al*., 2002). Transcription factors null alleles $mef2^{22-21}$, tin^{346}, bin^{22}, and $minc^{A388}$ have been described previously (Azpiazu and Frasch 1993; Bour *et al*., 1995; Zaffran *et al*., 2001; Ruiz-Gomez *et al*., 2002). $In(1)z^{ae(bx)}$, z^{58g}, and $P\{w+LacZ\}Trl^{s2325}$ fly stocks were obtained from the *Drosophila* stock center (University of Indiana, Bloomington, IN). Additional information can be obtained from FlyBase (http://flybase.bio.indiana.edu). Embryos were staged according to Campos-Ortega and Hartenstein (1997).

Transvection Index

It is defined by the algorithm 1+ (E − C/E + C), where E is percentage of adults with normal wing position of the experimental genotype and C is the equivalent for the control genotype. Both genotypes are siblings. Index values range from 1 (maximal transvection) to 0 (minimal transvection). Wing position was determined in 4-d aged adults.

Histochemistry and Immunostaining

β-Galactosidase activity was assayed in larvae and adults of transgenic lines as described in Ashburner (1989) with minor modifications. Third instars were dissected and fixed in 1% glutaraldheyde in phosphate-buffered saline during 30 min. Adult thorax and abdomen muscles were fixed in 4% paraformaldheyde in phosphate-buffered saline for the same time. To detect variations on expression levels among different constructs, we monitored the blue reaction products for 1 h 30 min at 37°C in a humidified chamber. The highest expression level is attained at this incubation time. The numbers 0–3 indicate the relative intensity that each genomic fragment yields under these incubating conditions. Fragments showing no expression were confirmed by additional 24-h incubation. Inmunohistochemical staining was performed as described previously (Marques *et al*., 2002). Embryos were collected, dechorionized, and stained with anti-β-galactosidase (Cappel Laboratories, Durham, NC) at 1:1000 and biotinylated horse anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) at 1:200. Gene expression was monitored in whole mount in situ hybridizations with a digoxigenin-labeled RNA probe as described previously (Ruiz-Gomez *et al*., 1997). The RNA probe was generated from the L9-TnI cDNA template (Barbas *et al*., 1991). Homozygous mutant embryos were identified from balancer *LacZ* or GFP marked chromosomes.

Plasmid Vector, Transgenic Lines and Site-directed Mutagenesis

Genomic fragments were subcloned in pBluescript KSII+. The *i*ntron *r*egulatory *e*lement (IRE)-0.89 kb fragment was produced by PCR by using the 4.7-kb clone as template with the forward primer 5′-GCTCTAGAGACTTCGGCTATGTACACTGCG-3′ and the reverse primer 5′-GCCCTCGAGCAAGCGGAATGGAAAAACAG-3′. After amplification, the genomic fragment was digested by *Xba*I/*Xho*I, cloned into the P transformation vector, and verified by sequence analysis. Other fragments were obtained in similar ways by using primers whose sequence is available on request. For transgenic lines, we produced a new vector, YAL, derived from YES (Patton *et al*., 1992). YAL contains the *Antennapedia* promoter driving *LacZ* as reporter, *yellow* as transformation marker, and flanked by *suppressor of Hairywing* – binding site boundary sequences (Gerasimova *et al*., 1995). IRE and *u*pstream *r*egulatory *e*lement (URE) fragments were cloned into *Sal*I–*Xba*I–*Xho*I targets sites of YAL polylinker, keeping the native orientation relative to the basal promoter. Germline transformation was performed in *y w* embryos by standard procedures (Spradling and Rubin, 1982). Site-directed mutagenesis was performed using PCR (Horton, 1995) on IRE-1.7 and IRE-0.89 templates. The sequence of oligonucleotides used to mutagenize each DMEF2 site is available on request.

Quantitative RT-PCR Assays

About 20 individuals of each genotype were used for RNA extraction by TRIzol reagent (Invitrogen, Carlsbad, CA). A total of 2 μg of RNA was reverse transcribed into cDNA by using First Strand kit (Amersham Biosciences, Piscataway, NJ) and 0.2 μg of oligo(dT) per reaction according to manufacturer's instructions. As normalizing internal control, we used the 140-kDa RNApolII subunit encoding gene (Falkenburg *et al*., 1987). Exon-specific oligonucleotide primers for the two genes tested, *wupA* and *RNApolII*, were designed from the databank sequences by using the Primer Select (DNA Star) software, eliminating putative dimerizing pairs of primers. Primer sequences are available on request. Fragments were verified by sequencing. Reverse transcription products were used as template for PCR reactions by using several dilutions to generate the corresponding curves. The following products were included in the reaction: SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The amplification was carried out in a ABI PRISM 7700 sequence detector (Applied Biosystems) by using the following conditions: 2 min 50°C, 10 min 95°C, 40 cycles (30 s 95°C, 30 s 60°C, 30 s 68°C), and 1 cycle (15 s 95°C, 1 min 60°C, 1 min 95°C). To calculate the relative index of gene expression, we used the efficiency calibrated mathematical method (Soong *et al*., 2000). It is based on the algorithm Ratio = $[(\text{E ref})^{\text{Ct sample}}/(\text{E target})^{\text{Ct sample}}]/[(\text{E ref})^{\text{Ct calibrator}}/(\text{E target})^{\text{Ct calibrator}}]$, where (E ref) is the reaction efficiency for the *RNApolII* primers, (E target) corresponds to *wupA* gene, Ct sample is the average threshold cycle from the gene, the E value corresponding to the mutant genotype sample, and Ct calibrator is the average of the threshold cycles from the gene, the E value corresponding to the nonmutant genotype sample. In turn, the E values of each reaction are calculated from the standard curve slope according to $E = 10^{-1/\text{slope}}$ as described in Rasmussen (2001).

Western Blots

Total protein extracts were obtained from homogenized embryos. Monospecific anti-Troponin I (Barbas *et al*., 1993) and anti-Ariadne (Aguilera *et al*., 2000) antibodies were used at 1:1000 and 1:75 dilutions respectively. Signal was developed by the chemiluminiscent ECL method (Amersham Biosciences).

Electron Microscopy

Electron microscopy was performed as described previously (Therianos *et al*., 1995), with minor modifications. Embryos were dechorionated, devitellinized, and fixed with 6% glutaraldehyde in phosphate buffer (pH 7.2) for 5 h at room temperature. Six 10-min washes with phosphate buffer were followed by postfixation in 1% OsO_4 in the same buffer for 1 h at room temperature. Dehydration was in graded ethanol series and embedded in Epon 812 resin. Samples were stained in 2% uranyl acetate (dihydrate) in aqueous solution for

60 min, followed by 15-min lead citrate incubation. Ultrathin sections of 70 nm were mounted on Formvar-coated single slot grids and viewed on a JEOL 1200 EXII electron microscope at 80 Kv.

In Silico Sequence Analysis

Sequence analyses were based on EMBL/GENEBANK/DDBJ databases and carried out using the BLAST 2.0 programs of the National Center for Biotechnology Information. For putative transcription factors binding sites, we used MatInspector version 2.2 program in the TRANSFAC databases (Wingender *et al*., 2000) followed by BLAST analysis with reported consensus sequences.

RESULTS

Searching for Regulatory Elements

The gene *wings-up A* maps to an X chromosome region, 16F, whose genetic constituents are well characterized (Prado *et al*., 1999). We generated transgenic lines with genomic fragments that span the entire TnI encoding transcription unit. These fragments were inserted into a newly generated vector, YAL (see MATERIALS AND METHODS), whose flanking sequences are of the insulator type aiming to prevent local enhancer effects on reporter expression. Out of the four major fragments tested, only one yielded *LacZ* reporter signal, and it was specific for muscle tissue, including all muscle types (Figure 2, A and B). These observations indicate that downstream of the *wings up A* promoter, the only existing positive regulatory elements are those included within the referred 4.7-kb fragment that spans exons 1–5.

Muscle Expression of TnI Is Controlled by Two Elements

The positive regulatory elements detected in the 4.7-kb fragment were further restricted to a 2.9-kb subfragment that yielded the same expression pattern (Figure 2C). In the same way, the analysis of genomic regions upstream of the promoter region yielded an additional fragment with positive reporter activity that extended 2.5 kb and overlapped 500 base pairs with the previous element (Figure 2C). We refer to these two fragments as URE and IRE, respectively, to maintain the nomenclature as in other TnI genes (Yutzey *et al*., 1989). Both elements show identical muscle-specific patterns of expression at all times during development (Figure 3). The two regions, located between −2 and +2.5 kb with respect to the transcription initiation site, were further subdivided into smaller fragments and tested for reporter activity in transgenic lines (Figure 2C). The resulting expression patterns are summarized in Table 1 and reveal spatial and temporal domains of specificity. These regulatory domains, as in many other genes studied (Arnone and Davidson, 1997), may overlap with each other forming a regulatory landscape rather than a line of mutually independent stretches of regulatory DNA (see DISCUSSION). For somatic muscles, the smallest fragment that drives an expression pattern equivalent to that of the native gene is the 1.7 kb that is fully contained within the first intron of the gene.

Transcription Factor Binding Sites within IRE and URE Regions

To identify transcription factors involved in *wupA* regulation, we first tested available null mutants for known transcription factors on their effects on TnI expression in vivo. As previously described, DMEF2 is required for transcription of muscle genes (Lin *et al*., 1996; Stronach *et al*., 1999; Arredondo *et al*., 2001; Kelly *et al*., 2002), and the corresponding mutant embryos showed absence of TnI RNA in all muscle types (Figure 4). By contrast, in mutants for TINMAN (Azpiazu and Frasch, 1993; Bodmer, 1993), BINIOU (Zaffran *et al*., 2001), and MINC (Ruiz-Gomez *et al*., 2002), TnI expression is prevented in selected muscle types only.

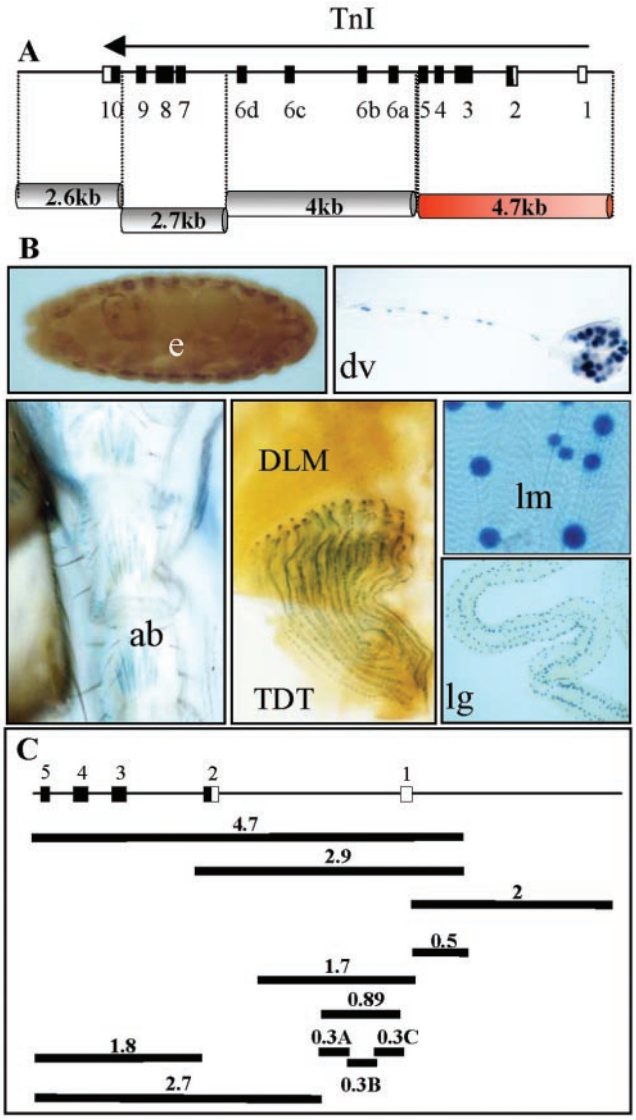

Figure 2. Genomic fragments from the *wings-up A* region and its reporter expression domains. (A) Extent in kilobases of major genomic fragments tested. (B) Expression domains of the only fragment that yielded expression, the 4.7 kb. Images of the expression in the embryo (e), dorsal vessel of the larvae (dv), adult abdominal muscles (ab), adult tergal depressor of the trochanter (TDT), dorsolongitudinal muscles (DLM), somatic larval muscles (lm), and larval gut (lg). (C) Genomic subfragments tested for reporter expression. Numbers indicate size in kilobases.

The first two seem to be required for visceral expression of TnI, and the third one is required only for somatic muscles.

An in silico analysis identified three putative DMEF2 binding sites within the IRE whose sequence seemed similar to that considered as consensus (Black and Olson, 1998) (e.g., consensus, YTAAAAATAR; site 1, TTAAAAATAC; site 2, TTAAAAATAA; and site 3, CTAAAAATG). These putative DMEF2 binding sites fulfill the muscle, as opposed to neural, sequence criteria (Andrés *et al*., 1995). To assay for their actual DMEF2 binding activity, we carried out in vivo and in vitro tests. Reporter transgenes of fragments containing normal and mutated versions of these sites were analyzed in all tissues and developmental stages (Figure 5 and Table 2). The significance of site 1 is indicated by the loss of reporter

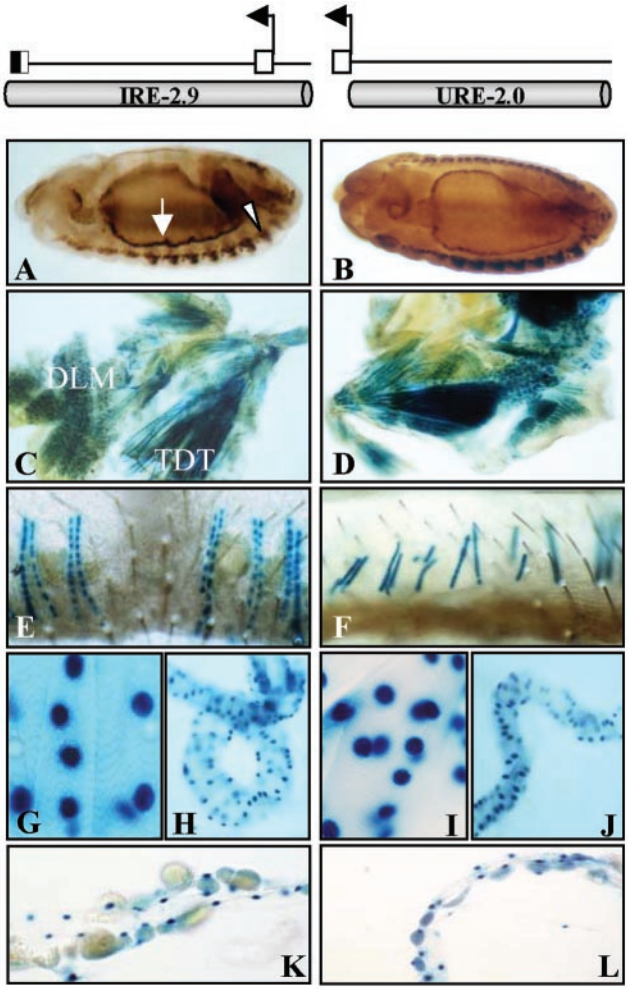

Figure 3. Identical reporter expression patterns from IRE and URE regulatory regions. (A and B) Reporter expression in somatic (arrow head) and visceral (arrow) muscles in the embryo. (C and D) Adult dorsolongitudinal (DLM) and tergal depressor of the trochanter (TDT) muscles. (E and F) Adult abdominal muscles. (G and I) Larval somatic muscles. (H and J) Larval midgut visceral muscles. (K and L) Larval heart. In all cases, reporter expression is revealed by β-galactosidase reaction in the cell nucleus. Top diagrams indicate the location and extent of fragments tested as transgenes.

expression in adult tergal depressor of the trochanter, dorsolongitudinal muscles, and dorsoventral muscles and a reduction in embryo somatic muscles when the IRE-0.89 fragment was assayed (compare Table 2, top two fragments). In addition, this site was tested for actual DMEF2 binding activity in band shift assays (see Supplementary Material). In the same way, site 2 activity is demonstrated by comparing reporter expression (see Table 2, second and third fragment) and band shift assays (see Supplementary Material). Finally, the activity of site 3 is supported by the remaining reporter activity of IRE-0.89 mut 2 fragment (Table 2). Mutations in the three sites (Table 2, lower fragment) yield a drastic reduction in all tissues, suggesting that the three sites act cooperatively. It is worth noting that although a null mutation in *Dmef2* abolished TnI expression in all muscle types (Figure 4A), a fragment with the three sites mutated still leaves a detectable expression in larval somatic muscles (Table 2 and Figure 5F) as well as in visceral muscles (our unpublished data). Because no other putative DMEF2 binding site was identified within IRE upon close inspection of its sequence, this weak signal should result from another transcription factor activity that is present at the larval, but not at the embryo, stage. This additional transcription factor would be required for basal maintenance, but not initiation, of transcription of this gene (see DISCUSSION).

The independent activity of URE as a regulator of TnI expression was documented in the initial screening where this region yielded an expression pattern identical to that of IRE (Figure 3). We analyzed its genomic sequence and found a number of transcription factor binding sites that occurred in a very similar arrangement compared with those in IRE (Figure 6, top diagram). The similarity includes the type of binding sites as well as their number and relative spacing. The apparent structural and functional redundancy between these two regions, however, proved misleading when the role of these regions in the expression of the native gene was studied (see below).

Conservation of the TnI Regulatory Array

For TnI genomic sequence comparisons, we chose another *Drosophila* species (*D. pseudoobscura*) and mosquito (*Anopheles gambiae*) because their genome sequences are available, and also those of rat (*Ratus norvegicus*) and quail (*Coturnix coturnix*) because in vitro functional data on regulatory regions are available. When comparing the genomic regions upstream and downstream to the promoter of insect TnI genes, there is a striking conservation of the type and array of putative transcription factor binding sites (Figure 6). For the rat slow, quail fast, and rat cardiac TnI genes, there are expression studies similar to those reported here, albeit in transfected cells (Yutzey *et al*., 1989; Banerjee-Basu and Buonanno, 1993; Nakayama *et al*., 1996; Murphy *et al*., 1997). With the exception of the rat cardiac gene, two regulatory regions upstream and downstream of the transcription initiation site have been documented. From the comparison between all TnI genes, it seems likely that the rat cardiac gene has lost the IRE region (Figure 6).

Considering other muscle genes in *Drosophila*, the available data on Troponin T- (Mas *et al*., 2004; this issue) and Tropomyosin 2 (Lin *et al*., 1996; Lin and Storti, 1997)-encoding genes show putative binding sites for the same transcription factors as in the TnI gene. In particular, the existence of two regions, URE and IRE-like, seems evident (Figure 6). Based on these observations, it seems that the *Drosophila* TnI regulatory arrangement is fairly well conserved in the orthologous counterparts and, possibly, in other genes encoding components of the muscle thin filament. The significance of this conservation is further supported by the unique nature of these regulatory regions. Three other genomic fragments tested for reporter expression and spanning ~12 kb distal to IRE failed to drive expression in any tissue or developmental stage (Figure 2A).

Testing the Native Gene Regulatory Mechanisms

The previous data were obtained by the procedure of dissecting the corresponding genomic fragments and testing their effect on the expression of a reporter gene. Here, we analyze the regulatory activity of URE and IRE on the native gene. To that end, we used three chromosomal rearrangements that delete URE [*Df(1)23437*] or increase the IRE-URE spacing [*P(w$^+$LacZ)PL87* and *P(w$^+$Gal4)PG31*]. The locations of the corresponding breakpoints were determined by plasmid rescue or genomic PCR (Figure 7A). The three rearrangements are lethal at the embryo stage, and their muscle phenotype is described further below.

Table 1. Reporter gene expression domains from IRE and URE subfragments

	Somatic muscles						Visceral muscles			Dorsal vessel			
	E	LIII	TDT	DLM	DVM	DFM legs	ABD	E	LIII	A	E	LIII	A
IRE-2.9	3	3	3	3	3	3	3	3	3	3	3	3	3
URE-2.0	3	3	3	3	3	3	3	3	3	3	3	3	3
IRE-1.7	3	3	3	3	3	3	3	1	3	3	0	0	0
IRE-0.89	2	3	0	0	0	3	3	1	3	3	0	0	0
IRE-0.3A	2	0	0	0	0	0	0	0	0	0	0	0	0
IRE-0.3B	0	0	0	0	0	0	0	0	0	0	0	0	0
IRE-0.3C	0	0	0	0	0	0	0	0	0	0	0	0	0
IRE-1.8	0	0	0	0	0	0	0	0	0	0	0	0	0
IRE-2.7	0	0	0	0	0	0	0	0	0	0	0	0	0
URE-0.5	0	0	0	0	0	0	0	0	0	0	0	0	0

Numbers indicate the relative intensity (0, no detectable signal; 3, maximal signal) of reporter gene expression under standard conditions. E, embryo; LIII, third instra larvae; A, Adult; TDT, tergal depressor of the trochanter muscle; DLM, dorsolongitudinal muscle; DVM, dorsoventral muscle; DFM, direct flight muscles; Legs, tubular leg muscles; ABD, abdominal somatic muscles.

The effects of these rearrangements on *wup A* expression were studied in Western blots and qualitative (Q)RT-PCR assays. In hemizygous *Df(1)23437* embryos, where URE is virtually eliminated, TnI is still detected by Western blot (Figure 7B). These data demonstrates that IRE is able to drive transcription independently from URE. The levels of expression, however, are insufficient for viability. In heterozygous females, this deletion reduces transcription, as indicated by QRT-PCR, to 50% (Figure 7B). The other two rearrangements were analyzed by Western blots in hemizygous embryos, by using the expression of another gene, *ari-1*, as an internal control, and normalizing the corresponding Ari-1/TnI levels to those of *Df(1)23437* (Figure 7C). Neither of the two rearrangements where URE is separated from IRE by ~11 kb, *PL87* and *PG31*, results in a null condition for *wup A*. These data demonstrate that IRE and URE are independently active in the native gene, albeit both must be within short distance to each other to synergize and yield full levels of transcription, and thus viability.

Transvection at the wupA Locus

The synergistic requirement of URE and IRE represent *cis*-interactions, presumably between enhancers and promoter. Chromosome pairing seems to be an additional requirement for proper transcription when two copies of the locus are present (i.e., in females, in this case) (Cook, 1997; Henikoff, 1997). In that context, we tested for transvection in heterozygotes between the three rearrangements, and between these and two point mutations in TnI. The latter correspond to $wupA^{hdp2}$, a A116V change in a constitutive exon (Beall and Fyrberg, 1991; Prado *et al*., 1995), and to $wupA^{hdp3}$, a single nucleotide change at the splice acceptor site for exon 6d (Barbas *et al*., 1993). In effect, allele *hdp3* can be considered a regulatory mutation because it leads to the absence of a subfamily of TnI isoforms, whereas allele *hdp2* is a structural mutation that does not interfere with transcription levels.

The homozygous genotypes for *PL87* or *PG31* result in viable and flying adults. This is the most efficient case of transvection observed at the *wup A* gene, and demonstrates that the deleterious *cis*-effects caused by the increased IRE-URE spacing can be repaired by an identical spacing in the homologous chromosome, presumably because chromosomal pairing at a critical region is now possible again. Similarly, the *trans*-combination between *PL87* and *PG31* (Table 3) yielded some viable adults with normal muscle structure, supporting flight in young as well as in 10-d aged individuals. It should be noted, however, that pairing between these rearrangements cannot be perfect along the inserted 11 kb because the transgene in each case differs, *LacZ* and *Gal4*. It seems that this mismatch is tolerated by the transcription mechanisms to the point of yielding near complete transvection. Combinations of either of these two rearrangements over *23437* remain lethal. Heterozygotes over allele *hdp2* showed strong evidences of transvection because a large fraction of individuals exhibit normal wing position (transvection index values close to 1; see MATERIALS AND METHODS) (Table 3). Heterozygotes over allele *hdp3* showed the same effect, although with somewhat reduced transvection index values in the case of *PL87* and *23437* heterozygotes.

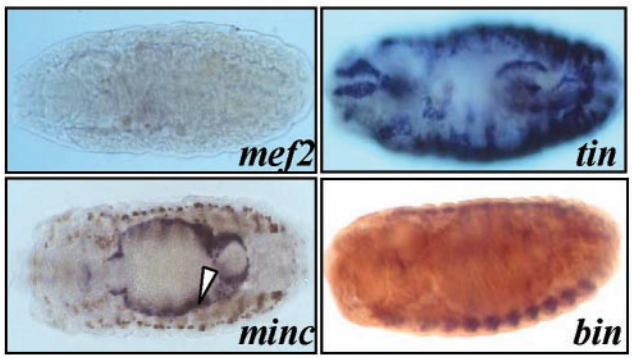

Figure 4. TnI expression in mutant embryos. Dorsal view of whole mount embryos degoxygenin stained for TnI RNA expression in homozygous mutant genotypes for the indicated genes. Note the lack of TnI expression in somatic and visceral muscles in the *mef2* null embryo, whereas *tinman* deficiency prevents expression in the heart and midgut only. Also, the *biniou* mutant prevents visceral expression only, whereas *minc* eliminates somatic expression. The dark signal observed in selected somatic muscle cells in the *minc* embryo (arrowhead) corresponds to an enhancer trap reporter within the *dumbfounded* gene that serves to mark the founder cells (Ruiz-Gomez *et al*., 2002).

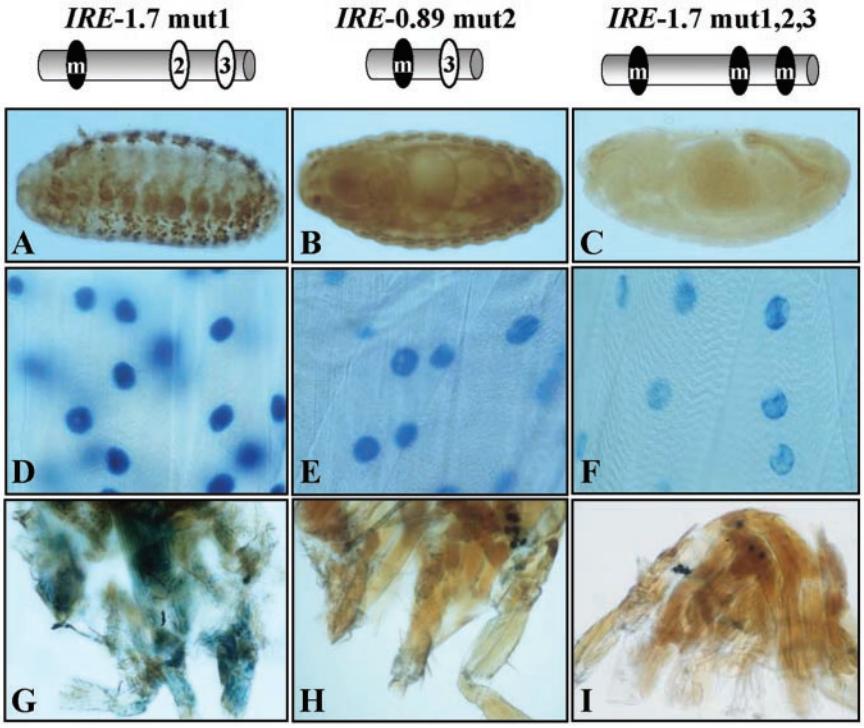

Figure 5. In vivo effects of MEF2 sites inactivation. Diagrams indicate the fragments extent, and status, mutated (black) or native (white) of each MEF2 site. (A–C) Transgenic embryos. Note total absence of signal at this stage when the three MEF2 sites are mutated (C), indicating that no additional MEF2 sites, active in the embryo, exist within the IRE fragment. (D–F) Somatic larval muscles. Note the progressive reduction of expression as individual sites are mutated, albeit maintaining a residual activity when the three sites are inactivated. These observations indicate that MEF2 is necessary for initiation, but not maintenance, of *wup A* basal transcription. (G–I) Adult leg muscles. Note the same effect of mutated MEF2 sites as in the embryo. MEF2 mutations are the same as in band shift assays (see Supplementary Material).

Transvection Is Dependent on zeste Activity

Transvection in other *Drosophila* genes is known to be influenced by DNA binding proteins involved in transcription and chromatin remodeling (Duncan, 2002; Kennison and Southworth, 2002). We tested the well characterized member of the Trithorax group of ubiquitously expressed cofactors encoded in *zeste* (*z*) (Laney and Biggin, 1996; Orlando *et al*., 1998; Hur *et al*., 2002), and the relatively less known *Suppressor of zeste 3 [Su(z)3]* gene. Two *zeste* mutant alleles were assayed in heterozygotes between *hdp* mutations and the three rearrangements (Table 3). The *zeste* background results in a reduction of transvection in virtually all genotypes tested (index values shift toward 0). The remarkable exception is the case of *PG31*. Heterozygotes over this rearrangement consistently maintain the highest transvection index. The observation seems to indicate that the location of this breakpoint, 249 base pairs upstream from the promoter, is beyond a critical distance for transcriptional effects in *trans* due to reduction of Zeste activity. Consistent with this interpretation, the shift of transvection index values is stronger in heterozygotes with *PL87*, which breaks at 30 base pairs upstream of the initiation site (see DISCUSSION). Heterozygotes including *Df(1)23437*, where URE is deleted, show the strongest shift yielding the minimum index value in most genotypes. Concerning the *wupA* alleles tested, *hdp2* shows consistently better transvection than *hdp3*, and seems virtually insensitive to either of the two *zeste* backgrounds.

In the case of the relatively uncharacterized *Su(z)3*, there is a strong reduction of transvection in heterozygotes involving the *hdp3*, but not *hdp2*, allele. The effect is strong enough as to be detectable in males (genotype: *hdp3*, *Su(z)3*/+). These become progressively unpaired to move and die in a few days. These observations suggest that *Su(z)3* play a role in *wupA* transcription similar to that of *zeste*.

Transvection Is Based on Transcriptional Changes

To know the molecular bases of the observed phenotypes, we measured transcription in various genotypes by QRT-PCR, discriminating among mRNA isoforms (Figure 8). The data confirm the reduction of exon 6d containing mRNAs due to the mutation *hdp3* (Figure 8A, first two histograms). In addition, the data show that a *zeste* mutant background elicits an increase of transcription. Because these QRT-PCR assays were done with exon 6d probes, the observed increase in transcription must originate from the wild-type chromosome only. Similar tests carried out with *Su(z)3* in males show that this gene acts, like *zeste*, as a repressor on *wupA* transcription (Figure 8B). Consistent with the shift of transvection index values and the enhanced severity of the motility trait in *hdp3* males, the transcriptional effect of *Su(z)3* seems stronger than that of *zeste*.

In a further attempt to correlate wing position phenotypes and transcriptional changes, we carried out QRT-PCR tests in flies sorted by wing phenotype, while identifying the chromosomal origin of TnI transcripts. The data show (Figure 9) that flies with normal wing position (evidencing transvection) yield higher levels of transcription than those with up wings position, in spite of being of the same genotype. This observation demonstrates that transvection is based on transcriptional changes. Furthermore, because the mutation hdp^3 prevents the generation of exon 6d-containing transcripts (Barbas *et al*., 1993), those detected here must come from the *Df(1)23437* chromosome. Consequently, their increased levels reflect a bona fide *trans*-effect on transcription.

TnI Is Required for Muscle Morphogenesis

The early expression of TnI, in addition to the severe transcriptional reduction caused by the three rearrangements, prompted a search for structural defects in muscle morphogenesis. Mutant embryos can be identified by genetic mark-

Table 2. Reporter expression from D-MEF2 mutated IRE fragments

	Somatic muscle						
	E	LIII	TDT	DLM	DVM	DFM legs	ABD
IRE-1.7	3	3	3	3	3	3	3
IRE-0.89	2	3	0	0	0	3	3
IRE-0.89 mut 2	2	2	0	0	0	0	3
IRE-1.7 mut 1	3	3	3	3	3	3	3
IRE-1.7 mut 1,2,3	0	1	0	0	0	0	0

Reporter expression levels (0–3) from IRE fragments where the D-MEF2 binding sites have been mutated (black) or not (white). The mutated versions were as follows: site 1: TTAAAAATAC → TTAAGCCTAC; site 2: TTAAAAATAA → TTAAGCCTAA; and site 3 CTAAAAATG → CTAAGCCTG. Fragments extent is indicated in kb and ordered with respect to the promoter (to the right).

ers (see MATERIALS AND METHODS) and subject to electron microscopy (Figure 10). The two mutants analyzed, *Df(1)23437* and *PL87*, show remnants of somatic muscles with poorly oriented thin filaments that never attain a sarcomere-like aspect. One consistent feature of these filaments, however, is the presence of regularly spaced electron-dense accumulations reminiscent of Z disk fragments. In the relatively less extreme phenotype of *PL87*, these fragments may look aligned as to form a proto-Z disk. Spacing between these proto-Z discs is the regular 80 nm, suggesting that proteins that facilitate the antiparallel organization of thin filaments and mark sarcomere dimension (i.e., kettin) are normally incorporated (Van Straaten *et al.*, 1999; Kulke *et al.*, 2001). In the more extreme phenotypes, however (Figure 10A), proto-Z discs begin to form at much shorter intervals, indicating that incorporation of other protein components to thin filaments is not a prior requirement for Z discs formation.

To further document the possible involvement of TnI in Z disk morphogenesis, we analyzed adult indirect flight muscles of genotypes in which a defined (Ala115Val) structural

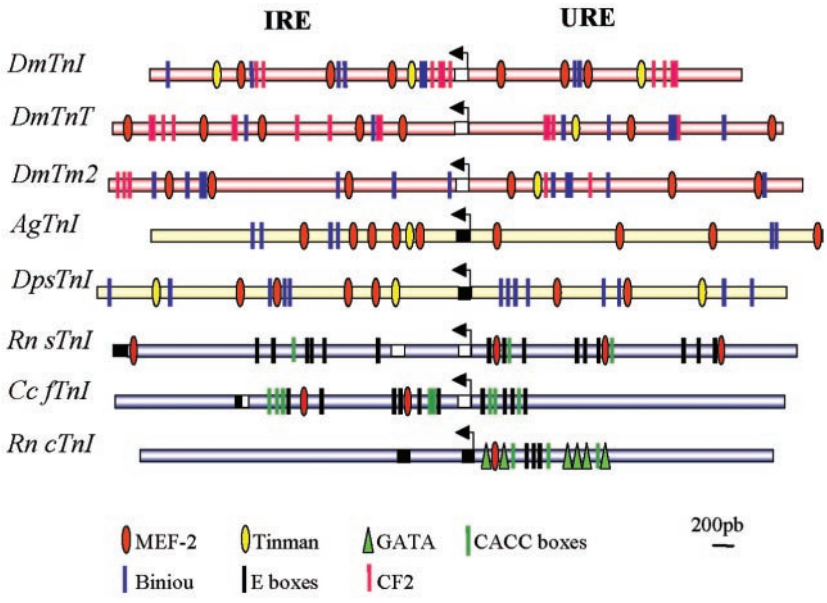

Figure 6. Conservation of the TnI regulatory array. Diagrams of three *D. melanogaster* genes (TnI, TnT, and Tm2), TnI from *D. pseudoobscura* and *An. gambiae*, and three vertebrate genes encoding slow, fast, and cardiac TnI, respectively. As with the IRE and URE regions of *wupA*, all these genes exhibit similar clusters of transcription factors, binding sites upstream and downstream of the transcription initiation site (arrow in each diagram). For *D. melanogaster* TnT gene, an independent demonstration of IRE and URE regulatory regions is also available (Mas *et al.*, 2004; this issue). Also, for *D. melanogaster* Tm 2, an IRE-like region has been experimentally demonstrated (Lin and Storti, 1997). Finally, for vertebrate's TnI genes, with the exception of the cardiac case, there is also experimental evidence of regulatory activities upstream and downstream of the initiation site (Banerjee-Basu and Buonanno, 1993; Nakayama *et al.*, 1996; Murphy *et al.*, 1997). The accepted binding site sequences for *Drosophila* and *Anopheles* have been MEF2, YTAAAAATAR (Black and Olson, 1998); TIN, TYAAGTG (Gajewski *et al.*, 1997); BIN, RTAAAYA (Zaffran *et al.*, 2001); and CF2, RTATATRTA (TRANSFAC databases at www.gene-regulation.com; Wingender *et al.*, 2000). Coding (black) and noncoding (white) exons are indicated as boxes.

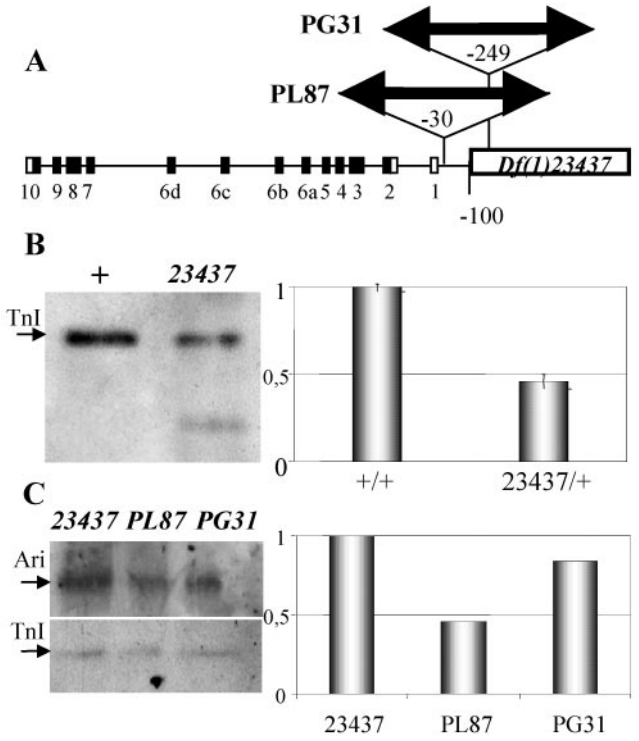

Figure 7. Expression effects of rearrangements. (A) Diagram indicating the location of the three rearrangements used here. *PL87* and *PG31* are insertions of about the same length (see MATERIALS AND METHODS) located at positions –30 and –249 base pairs with respect to the transcription initiation site. *Df(1)23437* is a 2-kb deletion located at position –100. Coding (black) and noncoding (white) exons are indicated as numbered boxes. (B) Western blot (left) from normal (+) and mutant (*23437*) embryos revealing that TnI is still expressed, albeit reduced, when the URE region is deleted, demonstrating that IRE can drive TnI expression independently from URE. Transcription levels are demonstrably reduced by QRT-PCR in heterozygous mutant adults (right). Transcription was normalized with respect to RNA polymerase II. Error bars indicate average SE from three independent RNA extractions. (C) Western blot (left) from mutant embryos showing TnI expression with respect to that of Ariadne-1 (Ari) used here as an internal control. This blot is shown as a quantitative densitometry (right) to illustrate the effect of *PL87* and *PG31* where IRE and URE have been separated but remain intact. None of the rearrangements are null. Data normalized with respect to the Ari-1/TnI ratio in *23437* embryos.

mutation in TnI, hdp^2, is coupled to either a second structural mutation (Leu188Phe) in TnI, *D3* or to an independent structural mutation (2-base pair insertion at exon 7) in the myosin heavy chain, *D41*. These second mutations suppress almost completely the functional defects of hdp^2 and show normal levels of gene expression (Prado *et al*., 1995; Kronert *et al*., 1999). In both genotypes, Z discs can be found with subtle abnormalities, including failures in their ordered alignment or failure to anchor all thin filaments from a sarcomere (Figure 10, C–F). Together, these data suggest a developmental role for TnI in Z disk that could result either directly from a structural role of TnI in thin filament morphogenesis, or indirectly from the down-regulation of Z disk constituents.

DISCUSSION

We address here the transcriptional mechanisms of the TnI encoding gene of *Drosophila*. This in vivo study reveals two regulatory regions, URE and IRE, located immediately upstream and downstream to the transcription initiation site and defined by a characteristic array of binding sites for the transcription factors DMEF2, BINIOU, and TINMANN. The regions are qualitatively identical in their effects, but both are required for proper levels of transcription. Given the span of the genomic fragments tested for the reporter expression, it seems that the full set of positive regulatory elements has been identified. Putative repressor sites, however, remain to be identified. Finally, the transvection experiments suggest that, in addition to the *cis*-requirements, transcription is also dependent on *trans*-effects occurring probably at a small critical region close to the putative promoter.

Table 3. Transvection index in *wings up A*

Genotype	Transvection index
PG31/PL87	1
PG31/hdp^2	1
PL87/hdp^2	1
23437/hdp^2	1
PG31/hdp^3	1
PL87/hdp^3	0.7
23437/hdp^3	0.6
Zeste	
PG31/hdp^2z^{58g}	1
PL87/hdp^2z^{58g}	0.9
23437/hdp^2z^{58g}	1
PG31/$hdp^2z^{ae(bx)}$	1
PL87/$hdp^2z^{ae(bx)}$	0.9
23437/$hdp^2z^{ae(bx)}$	0.9
PG31/hdp^3z^{58g}	1
PL87/hdp^3z^{58g}	0.4
23437/hdp^3z^{58g}	0
PG31 $hdp^3z^{ae(bx)}$	1
PL87/$hdp^3z^{ae(bx)}$	0.8
23437/$hdp^3z^{ae(bx)}$	0
Suppressor (z)3	
PG31/hdp^2*; Su(z)3/+*	1
PL87/hdp^2*; Su(z)3/+*	1
23437/hdp^2*; Su(z)3/+*	1
PG31/hdp^3*; Su(z)3/+*	1
PL87/hdp^3*; Su(z)3/+*	0.2
23437/hdp^3*; Su(z)3/+*	0

Maximum transvection is indicated by value index 1 and minimum by 0. Index algorithm (see MATERIALS AND METHODS) was determined according to wing position in four days aged adults. All *PL87/PG31* flies exhibit flight ability.

Regulatory Modules in wings up A

The genomic fragments analyzed in *LacZ* reported transgenes allow identifying regions that contain positive regulatory elements that direct expression to specific tissues and developmental stages. These regulatory modules are revealed as overlapping stretches of DNA rather than separate and mutually exclusive units. For example, the modules for somatic and visceral muscles share ~1 kb of sequences. None of the smaller fragments tested that subdivide this 1 kb, however, could reproduce the original somatic or visceral expression patterns. The case has precedents in other genes such as *mef2* (Cripps *et al*., 1999; Nguyen and Xu, 1998), *tubulin* (Damm *et al*., 1998; Kremser *et al*., 1999), or *tropomyosin 2* (Lin *et al*., 1996; Lin and Storti, 1997), and illustrate the intimate relationship between specific se-

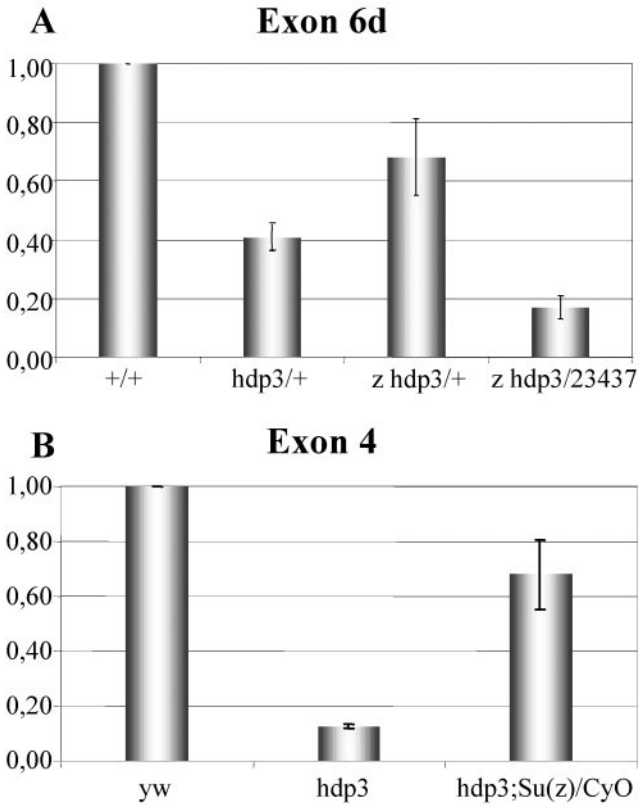

Figure 8. Transcriptional effects of *z* and *Su(z)3*. QRT-PCR assays on *wupA* expression in various genotypes. Transcripts were detected with primers from the alternative exon 6d (A) or constitutive exon 4 (B) and normalized with respect to the parental strain *y w* by using RNApolII as an internal standard. Error bars indicate average SE from a minimum of three RT-PCR assays.

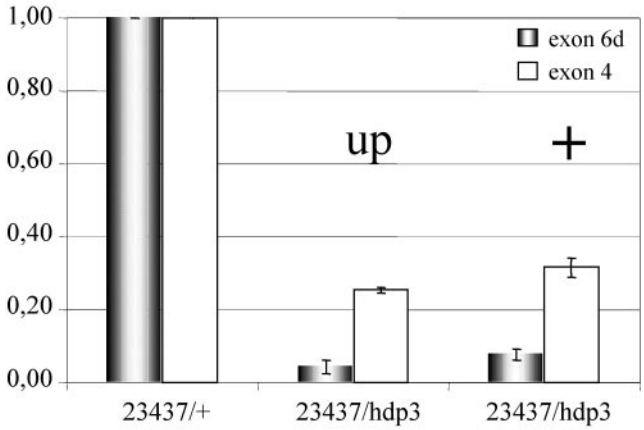

Figure 9. Relationship between phenotype and transcription in transvection. QRT-PCR assays in a given genotype that exhibits transvection and, in addition, allows identifying TnI transcripts produced by *Df(1)23437*-bearing chromosome. The mutation *hdp3* prevents the generation of exon 6d containing transcripts, whereas exon 4 reveals all transcripts. Four days aged adult females were sorted according to wing position indicative of transvection (+) or not (up). Note the increased transcription in the transvecting flies (+) with respect to those not exhibiting the phenomenon (up). The levels of exon 6d-containing transcripts must come from the *23437* chromosome, thus demonstrating a bona fide *trans*-effect. Error bars indicate average SE from a minimum of three independent RNA extractions.

quences (i.e., enhancers or repressors) and the topology of the surrounding chromatin in the context of proper gene expression (Yuh *et al*., 2001).

Mechanism of IRE + URE Activity for Transcription

The location of the two regulatory regions could sustain a particular chromatin structure, perhaps of a hair-pin type, for normal transcription. The spacing requirement and the linear order of interference effects shown by the three rearrangements support this speculation. In females, normal transcription requires correct pairing between both IRE + URE complements, and the spacing becomes less critical as long as it is the same in both chromosomes. The transvection effect that takes place when two homologous copies of the gene are present clearly implies enhanced transcription from the *trans*-homologue, as demonstrated by QRT-PCR assays in genotypes that allow to discriminate the chromosomal origin of some transcripts (Figure 9).

The initiation of transcription is clearly dependent on DMEF2. Maintenance, however, does not seem to rely exclusively on this transcription factor. The difference between these two types of transcription has been recently documented (Wheeler *et al*., 2002), and the present case may indicate that it is a general phenomenon. Because the analyzed regions contain canonical binding sites for TINMAN and BINIOU, it seems puzzling why these factors are not able to drive gene expression in a *mef2* null background. One possibility is that DMEF2, in addition to its direct DNA binding activity on *wupA* and its role as a transcription factor, acts also as a trancriptional cofactor for TINMAN and BINIOU. Also, the role of other transcription factors such as the one encoded in *Dmeso18E* (Taylor, 2000) remains to be integrated into this scenario. Purification and analysis of the corresponding protein complexes would be required to test these speculations.

Concerning the Trithorax group of transcriptional cofactors assayed, the data demonstrate that *zeste* acts as a repressor for *wupA*. In addition, we show that *Su(z)3* is also a repressor and it behaves similarly to *zeste* with respect to transvection effects. Additional data on a third gene, *Trithorax-like*, which encodes the GAGA factor yielded similar effects (our unpublished data). They represent *cis*- and *trans*-requirements for normal transcription. There seems to be, however, a critical domain near the promoter where the effects of these repressors become evident. Based on the immunity of *PG31* heterozygotes to Trithorax group mutant backgrounds, this critical region could be defined by the –249 position as the upstream limit. In addition, the perfect transvection in *PL87/PG31* heterozygotes suggest also a critical region of pairing for transcription. It is plausible that both critical regions are coincident. Presumably, pairing of these two rearrangements will be facilitated by the common sequences and size of the inserts. Their different site of insertion and the different transgenes, however, most likely will distort pairing to some extent. Thus, the critical region for transvection might be as small as 30 base pairs upstream of the initiation site. Extensive studies in the gene *yellow* have reached the same conclusion where the critical region for transvection seems to be the TATA box and an initiator element located in *cis* (Morris *et al*., 1999). The case of *wupA*, which does not contain TATA box, suggests that the critical region is the promoter per se, independently of its type. These observations should help to direct future in vitro studies with chromatin fragments.

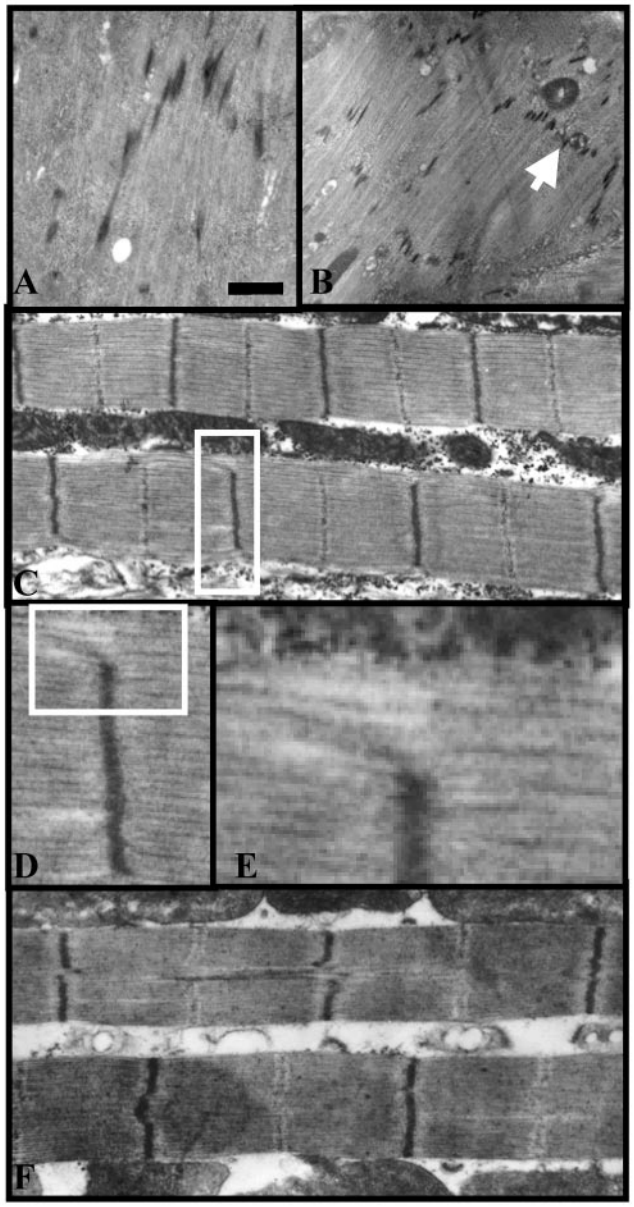

Figure 10. Muscle phenotypes of *wup A* mutants. Electron micrographs of mutant somatic muscles. (A) *Df(1)23437* embryo showing thin filaments with putative Z disk electrodense material at random locations along the filaments. (B) *PL87* embryo with better organized parallel thin filaments and a lined up proto Z disk (arrow). (C) Adult indirect flight muscles with mutated Troponin I and Myosin heavy chain proteins (genotype: males *wup* A^{hdp2}; Mhc^{D41}/+). Note the nearly normal sarcomere, albeit some thin filaments bypass a Z disk and continue to the adjacent sarcomere (inset). (D) Detail of inset in C. (E) Detail of inset in D. (F) Adult indirect flight muscles with double mutant TnI (genotype: males *wup* A^{hdp2} *wup* A^{D3}). Note the abnormal central part of some Z discs. Bar, 0.5 μm (A and D); 0.25 μm (B and E); 1 μm (C and F).

Wing Position Phenotypes and Transcriptional Changes

It may seem counterintuitive the observation that z and *Su(z)3* mutant backgrounds result in an increase of transcription at *wupA*, whereas the phenotypic effect shows a loss of transvection. Because the transcriptional change has been consistently observed in all genotypes assayed, including the flies sorted by wing position, it is evident that the phenotype does not correlate with the absolute levels of TnI transcripts. Furthermore, in spite of the very low levels of transcription in *23437/*hdp^3 females, they are viable and muscles are functional, except those involved in flight. The most plausible interpretation of these observations is that the deleterious effect on muscle structure results from changes in the stoichiometry of certain TnI isoforms rather than in their absolute levels (Gunthorpe *et al*., 1999). It is likely that, as in TnI isoform replacement experiments with the vertebrate homologues (Metzger *et al*., 2003), the unbalance of certain TnI isoforms lead to unsuitable thin filaments in vivo. If this is also the case in humans, certain muscular diseases, particularly those revealed under intense exercise, may result from mutations in regulatory regions, and thus may have escaped detection under standard sequencing procedures. In this context, the conserved gene regulatory array should be useful to guide future mutant screenings in humans.

Z discs are thought to be the anchoring points where thin filaments exert force and contract the sarcomere during muscle activity. It is somewhat unexpected that reduced levels or structural modifications of TnI can result in defective Z discs. The aspect and spacing of these Z disk-like structures suggest that a Z disk results from the lining of independent substructures deposited on the thin filaments and latter organized in register. It is worth noting, however, that a thin filament does not necessarily anchor at a Z disk. The quasi-normal sarcomeres from double mutants in troponin I, hdp^2, and myosin or tropomyosin, *Su(hdp2)* (Figure 10), show frequent cases of thin filaments extending more than one sarcomere in length. All these abnormal features of Z discs observed in mutants that involve TnI may indicate additional developmental functions of this protein beyond the well known regulatory role in sarcomere mechanics. Alternatively, these features may result from depletion of *bona fide* Z disk components whose expression is down-regulated because of TnI mutations. This possibility would require a coordinated regulation of gene expression among thin filament components.

A Conserved Regulatory Scenario: Biological Significance

Vertebrate TnI encoding genes are not yet amenable to the in vivo analysis that *Drosophila* allows. Nevertheless, previous studies on the quail fast and slow TnI genes show functional evidences of a very similar regulatory structure to that described here for *wupA* (Yutzey *et al*., 1989; Banerjee-Basu and Buonanno, 1993; Nakayama *et al*., 1996). Equivalent regions to IRE and URE can be revealed by sequence analysis in other TnI members with the exception of the cardiac gene. The array of regulatory regions and their characteristic features seem fairly well conserved in TnI encoding genes of insects and vertebrates. This observation will be relevant toward the design of tools that aim to mimic the native gene expression in otherwise pathological conditions. Beyond this utilitarian use, the conservation of the TnI regulatory landscape in other genes that encode thin filament components might be indicative of common trends that would ensure proper quantitative expression of these components and, eventually, could help to translate gene regulation into physiology.

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