Transcription of \textit{Drosophila} Troponin I Gene Is Regulated by Two Conserved, Functionally Identical, Synergistic Elements

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The \textit{Drosophila} \textit{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 \textit{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 genes act as repressors for \textit{wings-up A}. Critical regions for transvection and for Zeste effects are defined near the transcription initiation site. After in silico analysis in insects (\textit{Anopheles} and \textit{Drosophila pseudoobscura}) and vertebrates (\textit{Rattus} and \textit{Coturnix}), the regulatory organization of \textit{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., \textit{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 angiotension 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 \textit{Drosophila} homologue. In addition, this study takes advantage of the fact that TnI in \textit{Drosophila} is encoded by a single gene, \textit{wings up A} (\textit{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 \textit{cis}-effects, transcriptional changes can be elicited in \textit{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 \textit{Drosophila}, the single gene \textit{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 \textit{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.
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).

MATERIALS AND METHODS

Fly Strains and Crosses

Df(1)34337 and the point mutations hdp2 and hdp6 were described previously (Beall and Flyberg, 1991; Barbas et al., 1993; Prado et al., 1995, 1999). Regulatory mutants PL87 and PC32 were provided by H.M. Bourbon (Bourbon et al., 2002). Transcription factors null alleles mof22-2, tin455, bair2, and minaC336 have been described previously (Azpiazu and Frasch 1993; Bour et al., 1995; Zaffran et al., 2001; Ruiz-Gomez et al. 2002). Int(1)X/Y, z18, and P[+]=LuZ/Try22-24 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).

Transcription Index

It is defined by the algorithm 1+ (E - C1/E + C), where E is percentage of adults with normal wing position of the experimental genotype and C1 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% glutaraldehyde in phosphate-buffered saline during 30 min. Adult thorax and abdomen muscles were fixed in 4% paraformaldehyde in phosphate-buffered saline for the same time. To detect variations on expression levels among different constructs, we monitored the blue reaction product 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. Immunohistochemical staining was performed as described previously (Marques et al., 2002). Embryos were collected, dechorionated, and stained with anti-β-galactosidase (Cappel Laboratories, Durham, NC) at 1:1000 and biotinylated horse anti-mouse IgG (Signet Al- drich, St. Louis, MO) at 1:200. Gene expression was monitored in whole mount in situ hybrids with a digoxigenin-labeled RNA probe as described previously (Ruiz-Gomez et al., 1997). The RNA probe was generated from the 1.8-kb hdp6 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 cloned in pBluescript KSII+ . The intron regulatory element (IRE)-0.89 kb fragment was produced by PCR by using the 4.7-kb clone as template with the forward primer 5'-GCTCTAGAAGCTTGGC- TATGTCACCTGGC-3' and the reverse primer 5'-GCCCTCGAGCAACGG- GAATCGGAAAAACAGC-3'. After amplification, the genomic fragment was digested by Xhol/XbaI, 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 hairy-wing – binding site boundary sequences (Gerasimova et al., 1995). IRE and upstream regulatory element (URE) fragments were cloned into Sall–Xhol–XbaI targets sites of YAL polynucleukar, 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) primer reaction according to manufacturer’s instructions. As normalizing internal control, we used the 140-kDa RNAPoll subunit encoding gene (Falkenburg et al., 1987). Gene-specific oligonucleotide primers for the two genes tested, wupA and RNAPoll, 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 (PE 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 (30s 95°C, 30s 60°C, 30s 66°C), and 1 cycle (30s 95°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 2^−[(E ref)−(E target)] sample (where (E ref) is the reaction efficiency for the RNAPoll 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/Ct sample 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 ( Aguiler a et al., 2000) antibodies were used at 1:1000 and 1:75 dilutions respectively. Signal was developed with 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, dehydrated, and embedded in 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% OsO4 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

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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/DDJB 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).

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), dorso longitudinal 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, CTAATAATG). 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
expression in adult tergal depressor of the trochanter, dor-solongitudinal 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 Tnl 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 Tnl 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 Tnl Regulatory Array

For Tnl genomic sequence comparisons, we chose another Drosophila species (D. pseudoobscura) and mosquito (Anophe-lellum gambiense) because their genome sequences are available, and also those of rat (Rattus 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 Tnl 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 Tnl 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 Tnl 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 Tnl 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 Tnl 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.
Similarly, the somal pairing at a critical region is now possible again. Homologous chromosome, presumably because chromosomal spacing can be repaired by an identical spacing in the gene.

The deleterious cis-alleles, whereas allele wup Ahdp2 is a structural mutation that does not interfere with transcription levels.

The homozygous genotypes for PL87 or 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.

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 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 wupa 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 (Figure 4).

Figure 4. TnI expression in mutant embryos. Dorsal view of whole mount embryos degoxyogenin 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).

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

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<tr>
<td>URE-0.5</td>
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</table>

Numbers indicate the relative intensity (0, no detectable signal; 3, maximal signal) of reporter gene expression under standard conditions. E, embryo; LIII, third instar larva; 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.

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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 hdp3 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-
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 Tnl in Z disk morphogenesis, we analyzed adult indirect flight muscles of genotypes in which a defined (Ala115Val) structural

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<th>DLM</th>
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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 → TTAAACCTAC; site 2: TTAAAAATAC → TTAAAGCTAA; and site 3: CTAAAATAC → CTAAAGCTTG. Fragments extent is indicated in kb and ordered with respect to the promoter (to the right).
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/123437 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 mutant embryos showing TnI expression with respect to that from three independent RNA extractions. (C) Western blot (left) with respect to RNA polymerase II. Error bars indicate average SE.

Table 3. Transvection index in wings up A

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<tr>
<th>Genotype</th>
<th>Transvection index</th>
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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 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.

**Regulatory Modules in wings up A**

The genomic fragments analyzed in LacZ reporter 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-
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 arrangement (Wheeler et al., 2002). 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 PLE87/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.
the abnormal central part of some Z discs. Bar, 0.5 μm. (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 A²hp2; MhcD41/+). 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 Tn1 (genotype; males wup A²hp2; wup A¹hp2). 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, includ-
REFERENCES


