

The Haplolethal Region at the 16F Gene Cluster of *Drosophila melanogaster*: Structure and Function

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ABSTRACT

Extensive aneuploid analyses had shown the existence of a few haplolethal (HL) regions and one triplolethal region in the genome of *Drosophila melanogaster*. Since then, only two haplolethals, 22F1-2 and 16F, have been directly linked to identified genes, *dpp* and *wupA*, respectively. However, with the possible exception of *dpp*, the actual bases for this dosage sensitivity remain unknown. We have generated and characterized dominant-lethal mutations and chromosomal rearrangements in 16F and studied them in relation to the genes in the region. This region extends along 100 kb and includes at least 14 genes. The normal HL function depends on the integrity of a critical 4-kb window of mostly noncoding sequences within the *wupA* transcription unit that encodes the muscle protein troponin I (TNI). All dominant lethals are breakpoints within that window, which prevent the functional expression of TNI and other adjacent genes in the proximal direction. However, independent mutations in these genes result in recessive lethal phenotypes only. We propose that the HL at 16F represents a long-range *cis* regulatory region that acts upon a number of functionally related genes whose combined haploidy would yield the dominant-lethal effect.

NULL mutations in most genes of diploid organisms appear recessive in heterozygotes because no phenotypes are detected under cursory inspection (Perrot *et al.* 1991). In this context, the reduced viability observed in heterozygous deletions of chromosomal regions is interpreted as a deleterious effect caused by the cumulative dosage reduction of independent gene products. However, the aneuploid analysis of *Drosophila* has shown several cases of relatively small deletions that are lethal in only one copy (Lindsley *et al.* 1972; Lefevre and Johnson 1973; Stewart and Merriam 1973; J. R. Merriam, B. Stewart, D. Yamamoto and R. Rahman, unpublished data). Table 1 shows all the regions known to show haplolethal (HL) effect. Two of them, 22F1-2 and 83D-E, correspond to the autosomes, while the other five are located in the *X* chromosome. The haplolethality of 22F1-2 results from null mutations at the *dpp* gene, which encodes a *Drosophila* homolog of the bone morphogenetic protein 2 (BMP-2; Padgett *et al.* 1987; Wharton *et al.* 1996). The case of 83D-E is unique in the genome because of its additional triplolethal (Tpl) condition (Lindsley *et al.* 1972). Four of the HL regions include a gene with muscle mutant phenotypes, leading to the proposal that haplolethality

might be based on the imbalance of interacting muscle or cytoskeletal proteins (Homyk and Emerson 1988). However, aside from the case of 22F1-2, the actual cause of haplolethality remains unexplained for all loci because a systematic genetic analysis of these regions has not been performed.

In the analysis of the K⁺ channel-encoding gene, *Shaker* (*Sh*), we found that the region contains several genes with *Shaker*-like mutant phenotypes. We named the region *Shaker*, after the first gene that was characterized in the area, and we consider, as a working hypothesis, that several genes in the region might be functionally related because of their phenotypic similarities. Among them, the gene *Frequenin* (*Frq*) encodes a Ca²⁺-binding protein (Pongs *et al.* 1993) that acts as a negative modulator of the *Shaker* K⁺ channels (Bourret-Poulain *et al.* 1994). Other mutants with chronic shaking of appendages and abnormal action potentials in the cervical giant fiber (CGF) neuron also mapped within the cluster (Ferrús *et al.* 1990). These mutations are distributed along 350 kb, in which the haplolethal at 16F is included (Tanouye *et al.* 1981; Ferrús *et al.* 1990). The HL was defined as the 170-kb interval between the breakpoints of *T(1;Y)W32* and *T(1;Y)V7* because the aneuploid *W32^o/V7^o* is lethal in heterozygous females. Furthermore, we showed that the muscle mutants *wings up A* (*wupA*) are included in this interval and correspond to the troponin I (TNI)-encoding gene (Barbas *et al.* 1991, 1993). At that point, it was not evident how many genes compose the cluster and which of them could be the cause of the haplolethal phenomenon.

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TABLE 1
The haplolethal regions of *D. melanogaster*

| Chromosome | Segment | Implicated genes ^a |
|------------|---------|--|
| <i>X</i> | 2B17-D1 | <i>fli-A</i> : α -actinin |
| <i>X</i> | 5A6-9 | <i>Mlc-c</i> : myosin alkali light chain |
| <i>X</i> | 7C5-9 | <i>mrr</i> : myosin rod related |
| <i>X</i> | 12A1-7 | <i>up</i> : troponin T |
| <i>X</i> | 16F1-8 | <i>wupA</i> : troponin I |
| <i>II</i> | 22F1-2 | <i>dpp</i> : BMP-2 |
| <i>III</i> | 83D-E | ? |

^aAlthough most of the HL regions contain a muscle/cytoskeletal protein, the direct relationship with haplolethality has been demonstrated only for BMP-2 and TNI. References are as follows: *fli-A*, Thomas *et al.* (1997); *Mlc-c*, Edwards *et al.* (1995); *mrr*, FlyBase (see also Lefevre and Johnson 1973); *up*, Epstein and Bernstein (1992); *wupA*, Barbas *et al.* (1991), and *dpp*, Gelbart (1989) and St. Johnston *et al.* (1990).

Two working hypotheses on the haplolethality of 16F were considered since the beginning of the genetic analysis of this cluster: (1) a single gene with strict quantitative requirements and (2) a combined depletion of functionally related gene products (Ferrús *et al.* 1990). In the first case, the HL phenomenon could be attributed to a single protein, as appears to be the case of BMP-2 for the 22F1-2 HL, or to a single regulatory mechanism that would control several independent genes. In the second case, however, the only type of mutations that would yield dominant-lethal phenotypes would result from major rearrangements that would inactivate the entire set of functionally related genes. To distinguish between these two alternatives and their variants, we attempted a more precise definition of the 16F HL by means of producing dominant-lethal mutations, aneuploids, and the transcriptional analysis of the region. Here, we report that the HL function corresponds to a 4-kb window of genomic DNA located toward the 3' end of the troponin I-encoding gene. Furthermore, it appears that the normal structure of this window is required for the correct expression of several adjacent genes whose products seem to be functionally related.

MATERIALS AND METHODS

Genetic procedures and nomenclature: With the exception of the new mutations and rearrangements described in this report, the mutants and rearrangements used are described in FlyBase, Lindsley and Zimm (1992), and Ferrús *et al.* (1990). The description of aneuploids between two *T(1;Y)*s are referred to as *D* for the distal element and *P* for the proximal element. Thus, *W32^p-V7^p* indicates the aneuploid obtained with the distal element of *T(1;Y)W32* and the proximal element of *T(1;Y)V7*. The *Dp(1;3)JC153* is the insertional element of *T(1;3)JC153*, whose breakpoints are 16E2-4, 17A12-B1, and 99D. This element was irradiated to obtain reduced versions, *Dp(1;3)JC153^R*, under the criterion of uncovering the heldup or outstretched wing phenotypes. For brevity, the duplicated element is referred to as *Dp*, and its reduced versions

are referred to as *R1-R6*. The nomenclature of the gene encoding troponin I is particularly confusing because it has been referred to in the literature as *heldup* (*hdp*), *wupA*, and *TnI*. In this report, we accept the FlyBase standards and use the *wupA* name when referring to the gene, and we use TNI when referring to the encoded protein. There is another locus, mapped at 17C, whose mutants have been also labeled with the name *heldup*. It was proposed that the lack-of-function mutations at this gene cause the heldup wing phenotype, while the excess-of-function mutations yield a dominant beadex phenotype (Lifschytz and Green 1979). The locus, however, appears to be rather complex (D. Segal, personal communication), and the present FlyBase standards use the designations *heldup-a* (*hdp-a*) and *heldup-b* (*hdp-b*) for that gene. Within the *Shaker* cluster, until the analysis allows a more precise definition, we use the provisional nomenclature of ME, V, and HL prefixes for each gene according to their location in the maternal effect, viable, or haplolethal regions, respectively (Ferrús *et al.* 1990). For mutagenesis, males with a *f^s os* or *wupA^{hdp2}*-marked *X* chromosome and the *Dp(1;3)JC153/TM3* constitution were X ray treated at a dose of 4000 rad (Philips MG 151 Be, 150 rad/min, 100 kV, 15 mA, and 2-mm Al filter). Mutagenesis with ethyl methanesulphonate (EMS), diepoxybutane, or ethyl nitrosourea were carried out as described in Ferrús *et al.* (1990).

Molecular biology procedures: Transgenic lines were obtained by the injection of a construct containing either of the three genomic fragments E4, E6, or E6L, or the troponin I L9 cDNA (described in Figure 5) under the control of a hsp70 promoter in a pW8 vector (Klomez *et al.* 1987). This vector carries a *w+* minigene as a transformation reporter. P-element-mediated germline transformation was performed according to described methods (Spradling and Rubin 1982). Southern analysis was done on genomic DNA extracted as described in Ausubel *et al.* (1991) and processed on nylon filters as described in Sambrook *et al.* (1989). For Northern blots, mRNA was extracted using the QuickPrep micro mRNA Purification Kit (Pharmacia, Piscataway, NJ) and processed as described in Ausubel *et al.* (1991). Quantitative analysis of *Sgs3* and *Sgs4* transcription was carried out on several autoradiograms with various exposure times (1–4 days) and quantified by densitometry in a 300A apparatus (Molecular Dynamics, Sunnyvale, CA). Polymerase chain reaction (PCR) tests were performed under standard conditions (Innis and Gel-fand 1990) using a DNA thermal cycler 480 apparatus (Perkin Elmer Cetus, Norwalk, CT). For genomic DNA PCR tests on the reduced versions of *Dp(1;3)JC153*, embryos of characteristic HL phenotype (Barbas *et al.* 1991) were obtained from the following cross: males *f^s os; Dp(1;3)JC153^R/Dp(1;3)JC153* \times females *T(1;3)JC153/FM6/TM1*. For reverse transcription (RT)-PCR tests, normal or mutant embryos were used and processed to obtain RNA by the isothiocyanate-phenol method (Chomzinsky and Sacchi 1987), as well as the corresponding cDNA using the first-strand cDNA synthesis kit (Pharmacia) and a mixture of random hexanucleotides. As primers, we used oligonucleotide sequences from exons 1 and 10 of TNI (Prado *et al.* 1995). The search for sequence homologies and their comparative analyses were carried out using the GCG programs from the University of Wisconsin (Devereux *et al.* 1984), FASTA (Pearson and Lipman 1988), and BLASTA (Altschul *et al.* 1990) on the major public data banks.

Dosage compensation analysis: The following genotypes were analyzed using their LIII salivary gland homogenates: (1) *Berlin*; (2) *Oregon-R*; (3) *wupA^{hdp2}*; (4) *wupA^{hdp3}*; (5) *wupA^{hdp2}*, *wupA^{D3}*; and (6) *f^s os; Dp(1;3)JC153/TM3*. mRNA was extracted from these genotypes and treated as in the Northern blots. Extracts from males and females were probed for *Sgs4* and *Sgs3* expression (Breen and Lucchesi 1986) and quantified

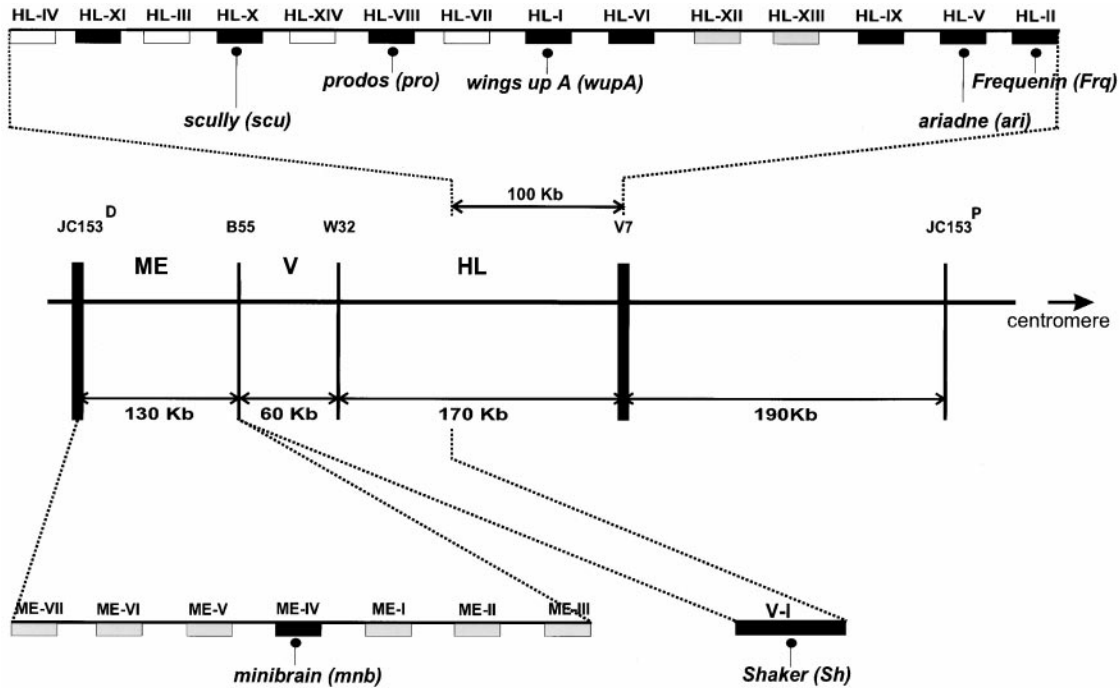


Figure 1.—The *Shaker* gene cluster. The distal and proximal (to the centromere) breakpoints of the insertional *T(1;3)JC153* serve to define a continuous stretch of 550 kb that has been cloned (Baumann *et al.* 1987) and used as the standard molecular map. The *Shaker* cluster corresponds to the most distal 360 kb and can be divided into three regions: ME, V, and HL by means of the *T(X;Y)B55*, *T(X;Y)W32*, and *T(X;Y)V7* breakpoints (Ferrús *et al.* 1990). The identified genes in each region, defined either as transcription units or complementation groups, are marked as roman-numbered boxes. Black boxes indicate genes for which mutants are available and a transcription unit is known. White boxes indicate genes known as transcription units without identified mutations. Shaded boxes indicate genes defined as mutant complementation groups that, to date, have no described transcription unit. Some of the best-characterized genes have definitive names such as *Shaker* (Baumann *et al.* 1987; Kamb *et al.* 1987; Tempel *et al.* 1987), *wings up A* (Barbas *et al.* 1991), *Frequentin* (Pongs *et al.* 1993), *minibrain* (Tejedor *et al.* 1995), and *scully* (Torroja *et al.* 1998). The genes *prodos* and *ariadne* correspond to our unpublished results. Centromere is to the right.

on a Molecular Dynamics 300A laser densitometer. A minimum of five determinations were taken for each genotype, and the average values were statistically compared by sex.

Histological procedures and embryo collection: The description of muscle phenotypes is based on 10- μ m sections of paraffin-embedded adults fixed with alcoholic Bouin's solution and stained with toluidine blue (Humason 1972). The age and criteria for the evaluation of phenotypes were used as described in Prado *et al.* (1995). Whole-embryo preparations for cuticle analysis were done according to the Hoyer method (Roberts 1986). Embryo staging was controlled by previous anesthesia with CO₂ of fecund females. After this treatment, females were placed into small population cages with removable fresh food trays. The trays were changed every hour, and the eggs were collected, dechorionated, and visually staged using standard criteria (Roberts 1986). For direct *in vivo* observation of embryogenesis, dechorionated embryos were placed under Voltalef oil and observed with dim light for several hours. For determination of the lethal phase, fecund females were allowed to lay eggs for 20-hr periods at 25°. Eggs were collected, counted, and placed on Petri dishes over filter paper soaked in 5% sucrose and a few drops of yeast. Groups of 20–30 eggs were examined at 1-day intervals to measure the rate of survival.

RESULTS

The *Shaker* region is a cluster of genes: The 16F-17A region of the X chromosome is currently the subject

of an extensive search for mutations and transcription units. The ongoing mutagenesis makes use of the 550-kb *Dp(1;3)JC153* to recover X chromosomes screened for lethal and viable phenotypes. Figure 1 is an update of the genetic map with respect to its previous version (Ferrús *et al.* 1990). At this time, the number of genes within the *Shaker* region that are defined either as complementation groups or transcription units is about three times higher than that in an equivalent extent of DNA outside of this gene cluster. For instance, the 170 kb corresponding to the HL region harbors 14 complete genes plus a good part (70 kb) corresponding to the *Sh* gene, while the adjacent 190-kb region between the *V7* and *JC153^P* contains only four genes (Eberl *et al.* 1992). Also, the current molecular analysis of the HL region confirms the high density of functions by revealing 12 transcription units (white and black boxes in Figure 1) so far. In the ME region, which spans 130 kb, the presence of seven complementation groups illustrates a case of medium gene density, although this region has not yet been analyzed in molecular detail. Thus, the present status of the analysis confirms that the *Shaker* region is a large mutational target because of the high density of functions with respect to the adjacent genomic interval.

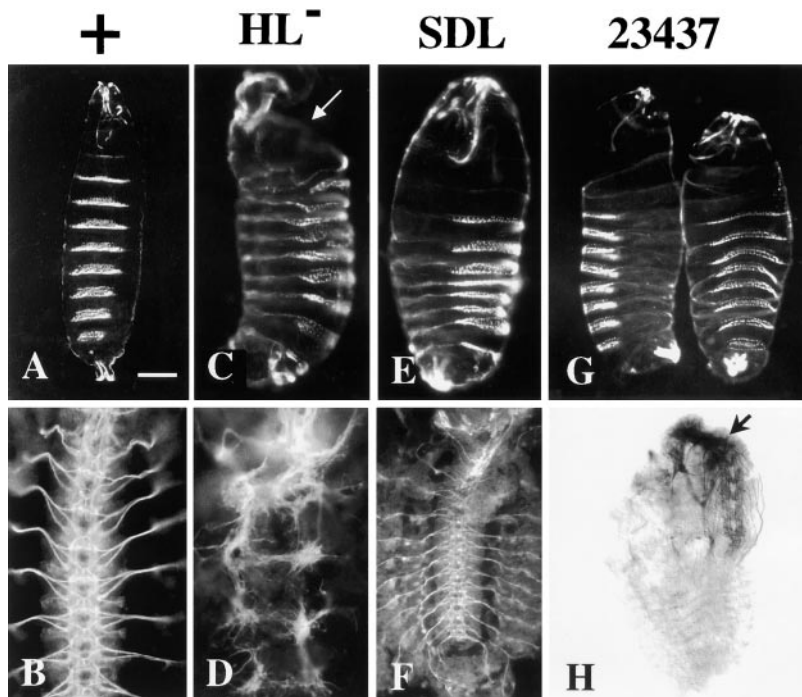


Figure 2.—Cuticular and neural phenotypes. The upper row (A, C, E, and G) shows the whole-mount cuticular structures in the wild type (+), *W32⁰-V7⁰* (*HL*⁻), semidominant lethal *8384* (*SDL*), and a recessive lethal in *HL-VI* (*23437*). The lower row (B, D, F, and H) shows the corresponding CNS phenotypes after Mab 22C10 staining. Note the similarity in the phenotypes, including the easily broken cuticle (arrow in C) and the extreme neural disarray in the cephalothoracic neuromeres (arrow in H). Bar, 20 μ m.

The *HL* region (*W32⁰-V7⁰*) is the main subject of this report because of its unusual genetic property of being haplolethal. The genetic manipulations in this region are made possible by *Dp(1;3)JC153*, while the identification of mutant embryos relies on the characteristic phenotype of deletions or the corresponding dominant-lethal mutations (see below). These mutant genotypes are lethal at the embryo stage with complete penetrance and expressivity, and they can be recognized because of their characteristic inflated, zeppelin-like appearance (Figure 2, A–D). Another striking feature is the aberrant structure of the central nervous system (CNS). Although the metameric organization of the CNS is still evident, most axons are misrouted and fail to fasciculate properly. The hypoderm differentiates normally, except in the ventral side of the cephalothoracic region, where the cuticle is so weak that it breaks under the coverslip pressure. The neural phenotype is also more extreme in this region of the body, as is frequently the case for many other embryonic mutations.

Dominant lethal mutations in the *HL* region result from chromosomal rearrangements only: Because the deletion of the interval *W32-V7* is haplolethal, lack-of-function dominant-lethal mutations must exist in this region. We used alkylating and ionizing agents as mutagens in the extensive search for mutations in this region (Ferrús *et al.* 1990). To date, only X-ray-induced mutations have been recovered with a dominant-lethal (*DL*) phenotype. In all cases, the *Dp* is required for viability. The *DL* mutations are equivalent to the *HL*⁻ deletion (*W32⁰-V7⁰*) because they have the same lethal phase and phenotype. Heterozygous *DL/+* females, as well as *HL*⁻/*+* female embryos, have a lethal phase that is

slightly more delayed than that of *DL* or *HL*⁻ males, and they show a milder version of the CNS phenotype (not shown).

To identify the chromosomal site for the haplolethal function, we characterized the molecular bases of the *DL* mutations. We analyzed the complete interval *W32-V7* in adult males of the genotype *DL ; Dp/+* by Southern blot. Figure 3 shows that the four *DL* mutations analyzed are rearrangements, with one of their breakpoints located between coordinates 198 and 203 of the standard genomic map of the area (Baumann *et al.* 1987). These breakpoints are the only aberrations detected in the interval of analysis. The nature of each rearrangement and the location of the second breakpoint are indicated in the legend of Figure 3. The simplest of them, *18242*, was sequenced and shown to be a 540-nucleotide insertion at position 10537 of the *wupA* genomic sequence (see below; accession number EMBL X58188), which corresponds to the intron between exons 7 and 8. We refer to this cluster of breakpoints as the *DL* domain.

In addition to the *DL* mutations, we found also two X-ray-induced mutations that yield a semidominant lethal (*SDL*) phenotype in *SDL/+* females (10–30% viability in outcrosses) with respect to sibling *SDL/+ ; Dp/+* controls. *SDL* males show an embryonic lethal phase with hypodermal and neural phenotypes quite similar to, although weaker than, the *HL*⁻ or *DL* genotypes (Figure 2, E and F). The *SDL/+* individuals that do not survive have their lethal phase at the embryo-LI stages, and they exhibit a further reduction in the severity of *HL*⁻ or *SDL* phenotypes. However, those individuals of the same genotype that survive to adulthood appear

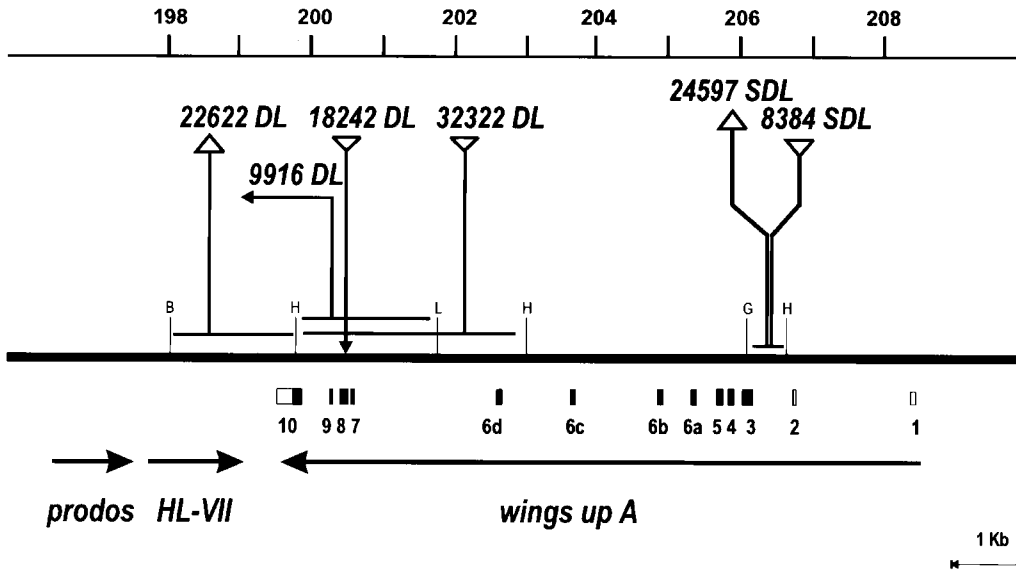


Figure 3.—Molecular map of dominant-lethal mutations. Four *DL* and two *SDL* mutations were mapped by Southern blot analysis. In all cases, the mutations result from rearrangements with at least one breakpoint in the TNI-encoding gene. Letters indicate the relevant restriction enzyme targets. B, *Bam*HI; G, *Bgl*II; H, *Hind*III; P, *Pst*I; R, *Eco*RI; L, *Sal*I. Horizontal lines between restriction sites (single-letter code) indicate the range of uncertainty in the location of each breakpoint. The standard map in kilobases is shown in the upper line. Arrows indicate the direction of transcription for

the genes in the area. Troponin I-coding (black) and noncoding (white) exons are indicated as numbered boxes. *9916DL* is an inversion with its second breakpoint located in division 14A. *18242DL* is an insertion of 540 bp. *22622DL* is a deletion of the chromosomal segment 16F-18D. *32322DL* is an insertion of ~3 kb. *8384SDL* is an insertion of ~8 kb. *24597SDL* is a deletion of ~0.4 kb.

normal in their morphology and cursory inspection of their behavior. Only a reduced body size is evident in *SDL24597/+*, but not in *SDL8384/+*, surviving females. After Southern blot analysis, both *SDL* mutations revealed breakpoints within a very narrow window around position 206 of the standard map between exons 2 and 3 of troponin I (Figure 3). The described Southern blot abnormalities in the *DL* and *SDL* mutants are the only ones detected in the *W32-V7* region. Thus, *DL* and *SDL* rearrangements appear to define two chromosomal domains with distinct phenotypes. No *DL* or *SDL* mutants have been isolated among >70,000 screened chromosomes mutagenized with the alkylating agents EMS, diepoxybutane, and ethyl nitrosourea.

The haplolethal function colocalizes with the TNI-encoding gene: The *DL* and *SDL* genomic domains correspond to the *wupA* gene (Barbas *et al.* 1991), in which the *wupA^{hdp2}* and *wupA^{hdp3}* point mutations yield viable

heldup wing phenotypes because of structural muscle defects in the adult (Barbas *et al.* 1993; Prado *et al.* 1995). The *DL* mutations demonstrate that this domain is necessary for a *HL⁺* function. To analyze which segment of DNA is sufficient to restore this function, we trimmed the *Dp(1;3)JC153* with X rays. The criterion to detect reduced versions, *Dp(1;3)JC153^R*, of this duplication was based in the uncovering of the wing position phenotype of *wupA^{hdp2}*. Each mutation known in the *W32-V7* interval and representative alleles of the ME complementation groups was tested for phenotype rescue with the isolated *R* duplications (Figure 4). A salient feature of this functional map is that all *R* duplications, selected for their loss of the *wupA⁺* function, have lost also the *HL⁺* property because none of the *DL* mutations are rescued by any of the *R* elements. The failure to rescue *DL* mutations is independent of the number of copies of *R* duplications or their pairwise combina-

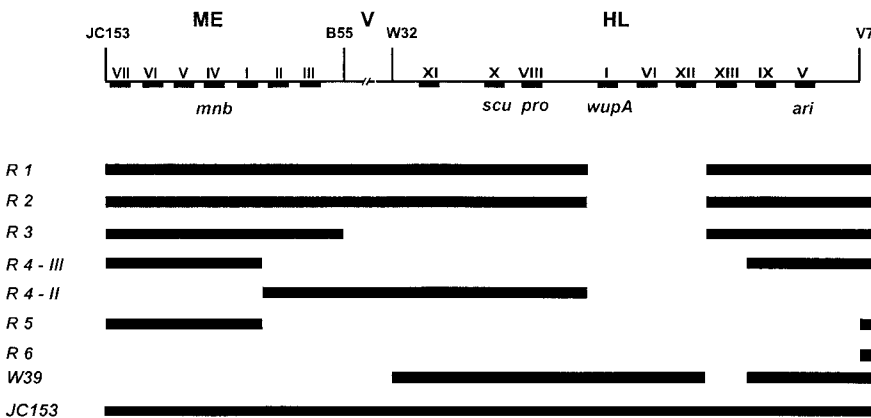


Figure 4.—Functional map of *Dp(1;3)JC153* derivatives. The reduced versions of the *Dp(1;3)JC153* were tested for rescue of lethality or visible phenotypes against representative alleles of each complementation group in males. The figure shows only the genes for which mutations are known and have been tested. Note that duplications *R1-R6* were identified by the criterion of uncovering the heldup wing phenotype, and, in all cases, normal *HL* function was also lost with *HLVI* and *HLXII* functions (see text). *Dp(1;Y) W39* is a derivative from *W32^R*.

tions, as well as the *DL* or *DL/+* conditions. Likewise, the *SDL* mutations are not rescued by the *R* duplications, although some of them rescue the lethality of *SDL/+* heterozygotes (see below).

The molecular location of *R* duplication breakpoints was determined by Southern blots probed with overlapping genomic fragments that cover the entire *W32-V7* interval. The most significant breakpoints, those of *R1*, *R2*, and *R4-II*, are indicated in Figure 5A. These duplications have a single break in the *W32-V7* region that coincides with the genomic domain previously defined by the *DL* mutations, the 3' half of the *wupA* gene. In addition, the integrity of the genomic DNA corresponding to 15 kb toward the proximal and 5 kb toward the distal directions from the *R1* and *R2* breakpoints was checked by PCR in embryos of the genotype *Df(1)JC153; R/+* using a collection of primers that span these intervals. The only primers that failed to amplify the proper DNA fragment were those corresponding to fragment 438E4 (Figure 5A). The *R1* and *R2* rearrangements are likely to have the second breakpoint outside of the *W32-*

V7 interval, but still within the *Dp(1;3)JC153*, because no cytogenetic alteration could be detected in polytene chromosomes of *R/Dp* salivary glands. The convergent results between *DL* and *R* rearrangements indicate that a domain of ~4 kb within the TNI-encoding gene harbors the HL⁺ function. In effect, the most resolving piece of data to locate the HL domain is the *18242DL*, which has been mapped to the nucleotide level. The smallest chromosomal fragment capable of rescuing *DL* mutations is *Dp(1;Y)W39* (Figure 4). This is a derivative from *W32^p* generated after X-ray treatment (Eberl *et al.* 1992) that we tested against all known mutations in the *W32-V7* interval.

TNI alone is not the cause of haplolethality: A number of arguments, derived from the previous characterization of the TNI-encoding gene (Barbas *et al.* 1991, 1993), fail to support this protein as the sole cause of haplolethality: (1) None of the 10 TNI isoforms is expressed outside of the muscle system, while the *DL* phenotype affects many tissues, including the nervous system and the hypoderm. (2) The *DL* mutants are lethal

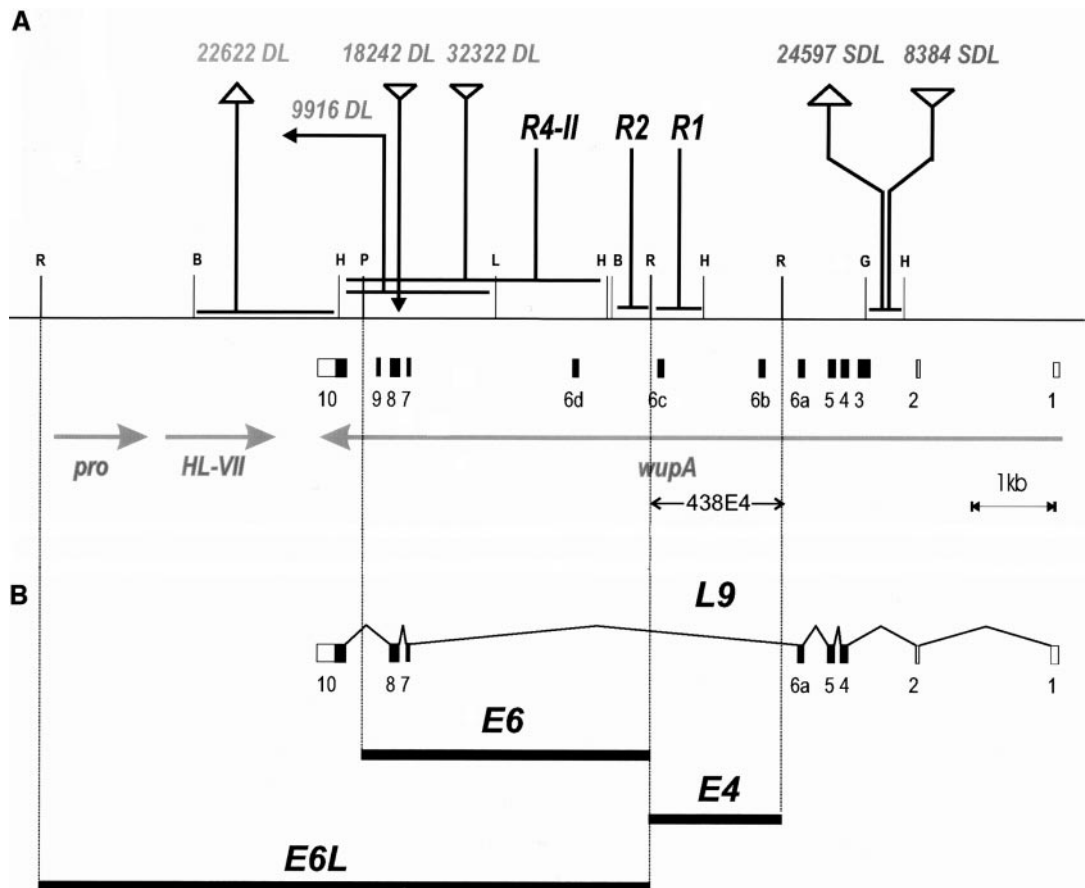


Figure 5.—Molecular map of *Dp(1;3)JC153* derivatives and extent of transgenic constructs. (A) Breakpoint location of the three most significant derivatives: *R1*, *R2*, and *R4-II*. Note that these breakpoints, in conjunction with those from the dominant-lethal mutations, help to restrict the HL region to an ~4-kb window within the TNI-encoding gene. The window is defined by the *HindIII* (H) and *EcoRI* (R) restriction sites located in exon 10 and intron 6c, respectively. The genomic fragment 438E4, defined by two *EcoRI* (R) sites, was not amplified from *R1* and *R2* derivatives by PCR. (B) Extent of the genomic fragments used for the transgenic lines E4, E6, and E6L. Also, the troponin I cDNA used, L9, is shown. None of these constructs rescue or modify the dominant-lethal phenotype. However, the fragment E6L rescues the lethal phenotype of a mutation in *prodos*.

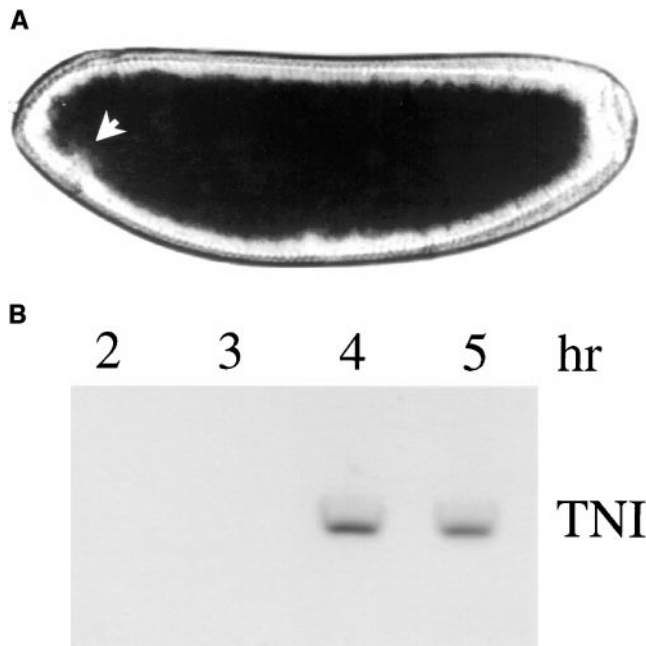


Figure 6.—RT-PCR analysis of *wupA* expression in the early embryo. (A) Mutant 9916*DL* embryo showing the earliest morphological phenotype. Many abnormal small invaginations of the peripheral yolk and internal protrusion of blastoderm cells can be seen, particularly in the cephalic region (arrow). All embryos exhibiting this trait develop the whole neural and hypodermal mutant syndrome at later stages. Anterior is to the left and dorsal is upward. (B) RT-PCR from carefully staged normal embryos of 2–5 hr of development (see materials and methods). The indicated developmental time in hours corresponded to the following visually determined stages: 2, stage 4; 3, stage 6; 4, stage 8; 5, stage 10. The samples were primed with oligonucleotides from exons 1 and 10 of troponin I.

in the germ line, while the TNI protein or its mRNAs are not a maternal contribution to the oocyte. (3) If TNI were the single cause of haplolethality, the *DL* rearrangements would have been distributed along the transcription unit instead of being clustered in a relatively small domain toward the 3' end. (4) TNI mutations, such as *wupA^{hdp3}*, which abolish the production of a subset of isoforms, would have been expected to be lethal instead of viable.

A further attempt to consider TNI as the single cause of the *HL* effect consisted in the generation of a transgene that expresses one of the *wupA* cDNAs, L9 (Figure 5B). We chose this cDNA because it is an isoform that is expressed early in development, when the *HL* phenotype becomes evident. Also, this cDNA has the longest untranslated region (UTR) at the 3' end (Decker and Parker 1995), and there is a precedent for a general regulatory function by a 3' untranslated region in the case of the vertebrate α -tropomyosin RNA (Rastinejad *et al.* 1993; L'Ecuyer *et al.* 1995). However, this transgene failed to rescue all the *DL* and *SDL* mutations. Incidentally, the transgene cannot rescue the via-

ble *wupA* mutations, most likely because their phenotype is detected in the indirect flight muscles of the adult, and we know that this tissue expresses other RNA isoforms (Barbas *et al.* 1993). In any case, we checked by Western blot that the transgene L9 is translated into the corresponding protein. Finally, we carried out a detailed time course of *wupA* expression during early development by RT-PCR. Figure 6 shows that transcription of this gene is first detected in 4-hr embryos, while the first mutant trait in *DL* or *HL*⁻ embryos is identified at 3 hr, before the onset of gastrulation. In view of all these results, albeit negative, we discarded the possibility that TNI, or a putative regulatory sequence at the UTR contained in the L9 mRNA, could be the single cause of the haplolethality, and we set out to search for other adjacent genes that could be implicated, perhaps jointly with TNI, in the haplolethal phenomenon.

The *HL* function requires a native *cis* location: We first considered the possibility of a hypothetical gene contained within an intron of *wupA* toward its 3' end, whose transcription would be eliminated by the *DL* mutations and the *R* breakpoints. This alternative was considered, even though the Northern blots probed with different genomic fragments of the area had failed to detect any RNA other than those for TNI. Also, the frequency of codon usage of either DNA strand had not suggested any additional open reading frames. Nevertheless, to test for the possibility of a small and difficult-to-track gene within an intron (Chen *et al.* 1987; Tycowski *et al.* 1996) of this transcription unit, we generated transgenes with genomic fragments, including the *DL* domain (Figure 5B). Several insertions of each of the three fragments, E4, E6, and E6L, were isolated, but none of them rescued any of the *DL* mutants. The most significant case is the 8-kb E6L fragment, which carries the normal function for *HL-VIII*. Transgenes with this fragment rescue the *prodos* mutants, but none of the *DL* or *SDL* mutants (D. Ortuño-Sahagún, C. Cases, A. Ferrús and J. A. Barbas, unpublished results). Other mutations in the region, particularly all available alleles of *HL-I*, *VI*, *XII*, and *XIII*, were also tested for rescue by the E6L fragment, with negative results further indicating that this genomic fragment cannot supply these normal functions when located in ectopic sites. The *HL-VII* could not be tested because no mutations are available for this gene. Thus, it appears that the *DL* domain needs to be located in *cis* with respect to some gene or genes that it would presumably regulate. Based on the location of the mutations that fail to complement *R1* and *R2* duplications (Figure 4), the *cis* effect appears to operate only in the proximal direction.

***wupA* probably has a functional relationship with adjacent genes:** An important finding of the functional characterization and the molecular localization of *R* breakpoints (Figures 4 and 5) is that all breaks in the *DL* domain, in addition to inactivating the TNI-encoding gene, also inactivate the two proximal functions *HL-VI*

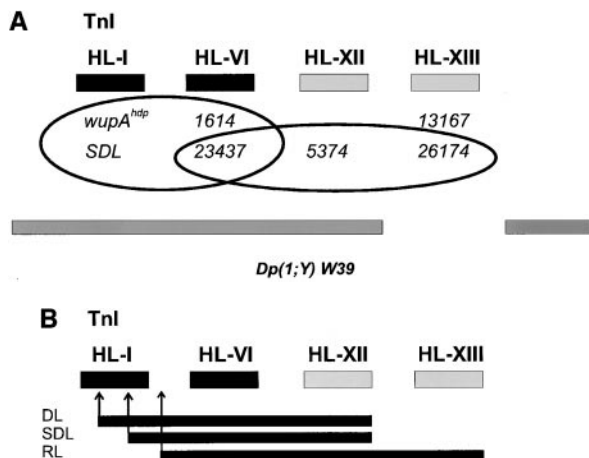


Figure 7.—Complementation analysis in the HL region. (A) Numbers indicate recessive lethal mutations. Circles include noncomplementing mutations. The *Dp(1;3)W39* fragment carries all the normal functions in the area, except those for 13167 and 26174. The failure of complementation between *HL-VI* and *HL-I* mutants is allele specific (see text). (B) Proposed extent of the regulatory effects by the DL, SDL, and RL domains.

and *HL-XII*. These genes are defined here as complementation groups and are represented by the recessive lethal mutations 1614 and 23437 on the one hand and 5374 on the other. The complementation map in this area shows three groups with overlapping patterns of complementation, indicated by ellipses in Figure 7. The Southern blot analysis of these three mutations manifests a rearrangement only for the X-ray-induced 23437, a 2-kb deletion located 100 nucleotides upstream of the transcription initiation site of *wupA*. This rearrangement, although recessive lethal, is also involved in the *HL* function (see below), and we refer to it as the RL domain. This deletion does not affect the expression of TNI in any form that we have tested, including Western

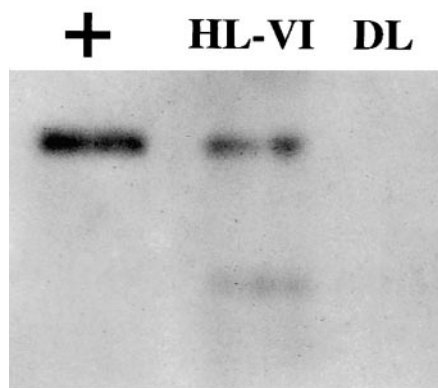


Figure 8.—Troponin I expression in *HL-VI* and *DL* mutations. Western blot from embryos of *CS* (+), 23437 (*HL-VI*), and 9916 (*DL*) genotypes hybridized with a monospecific anti-TNI serum. Note the lack of effect of 23437 upon TNI expression and the null condition of the *DL* mutation.

blot and RT-PCR assays (Figure 8). However, the transcription of the *HL-VI* unit is abolished in this mutant, as well as in its EMS-induced allele 1614 (our unpublished data). These two mutations for *HL-VI* fail to complement the semidominant lethality trait of *SDL* mutants (Figure 7), suggesting that *HL-VI* and *HL-I* could be functionally related. This suggestion prompted the test for complementation with *HL-I* point mutations, the *wupA* alleles. We analyzed the A116V mutation affecting all TNI isoforms, *wupA^{hdp2}*, and a splicing mutation that deletes a large subset of TNI isoforms, *wupA^{hdp3}* (Beall and Fyrberg 1991; Barbas *et al.* 1993; Prado *et al.* 1995). We find that 1614 fails to complement *wupA^{hdp2}* and *wupA^{hdp3}* with respect to wing position, flight, jump, and muscle structure (Figure 9). However, 23437 fully complements *wupA^{hdp2}*, but it shows an extreme phenotype over *wupA^{hdp3}* (Figure 9). This allele specificity in the complementation patterns suggested that the two mutations cause a differential molecular effect beyond their common null condition for *HL-VI* transcription.

To clarify the functional differences between 1614 and 23437, we analyzed the phenotypes at the corresponding lethal phases. While 1614 shows a distributed lethal phase along development, 23437 is a strict embryonic lethal. Also, 23437 embryos show hypodermal and neural phenotypes very similar to those of *DL* or *HL⁻* embryos (Figure 2, G and H), while 1614 yields occasional pharate adults with severe muscle defects (Figure 9). The muscle defects of 1614 indicate that the *HL-VI* function will be relevant to the biology of this tissue. Furthermore, the lack of complementation between *HL-VI* and *HL-I* (*wupA*) alleles support the proposal that these two products might be functionally related. The similar phenotypes of 23437 and *HL⁻* demonstrate that the mutation is a null equivalent to its deletion. By contrast, 1614 does not show these embryonic phenotypes. Because both mutations are transcriptional nulls for *HL-VI*, it can be concluded that 23437, but not 1614, affects an additional gene. This additional gene does not appear to be the one encoding TNI in view of the negative results mentioned above (Figure 8). According to the complementation map (Figure 7A), 5374 might represent this gene, *HL-XII*, and, thus, it would be the third component affected by the DL domain and the second one affected by the RL domain. A summary of the proposed hierarchy of control effects by the DL, SDL, and RL domains of the HL region is shown in Figure 7B solely for the purpose of serving as a working hypothesis. The inclusion of *HL-XIII* in the realm of action of the RL domain is suggested by the complementation analysis and should be considered only as a proposal at this time. The existence of this gene as an independent function, however, is proven by the *Dp(1;Y) W39*, which fails to complement both alleles of this group, but it does carry the normal functions for all other mutations in *HL-I*, *VI*, and *XII* (Figure 7A).

TNI is sensitive to dosage of other components of

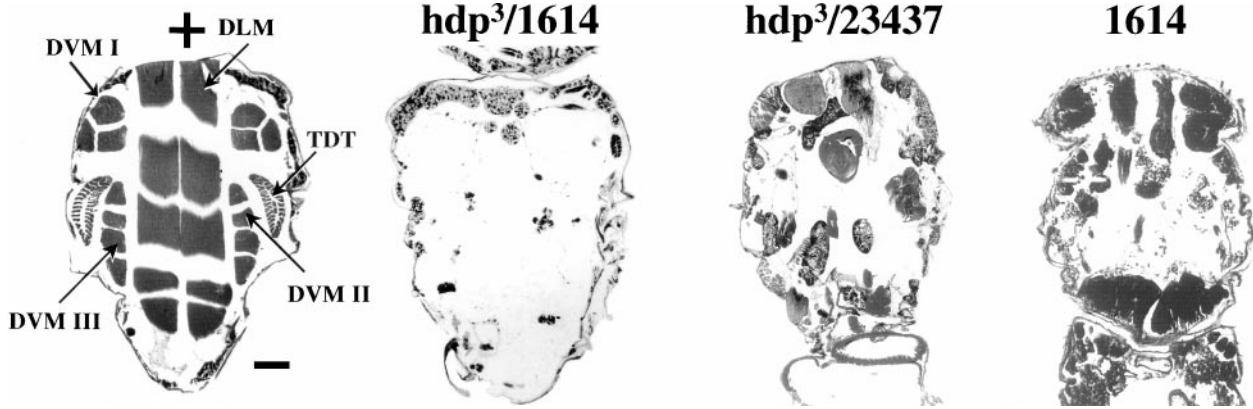


Figure 9.—Muscle phenotypes of *HL-I* and *HL-VI* mutants. Horizontal section of adult thoraxes. Canton-S female (+) shows the main muscles: dorsolongitudinal (DLM); dorsoventrals I, II, and III (DVM I-III); and tergal depressor of the trochanter (TDT). Noncomplementation between *HL-I* and *HL-VI* mutations is shown in double heterozygotes between *wupA^{hdp3}* and *23437*, *1614*. The pharate male *1614* mutant shows the direct involvement of *HL-VI* function in muscle development. Note the severe effects on all major indirect flight muscles in the three genotypes. These muscles are not affected in *23437*, *wupA^{hdp3}*, or *1614* heterozygotes (not shown). All cases are horizontal sections. Anterior is to the top. Bar, 100 μ m.

the cluster: During the course of the many crosses performed in this genetic analysis, we found that two doses of the *Dp* reduce the viability (Table 2). This concurs with the general observation that hyperploidies are not tolerated well (Lindsley *et al.* 1972). However, if the mutation *wupA^{hdp3}* is included in addition to the two copies of *Dp*, the genotype becomes lethal (Table 2). Similarly, if on a *wupA^{hdp2}* background, male and female flies are very poorly viable, sterile, and flightless (Table 2). This deleterious effect beyond the hyperploidy for *Dp* can be ascribed to the function of the TNI protein itself because the effect is reverted by a second mutation, *wupA^{D3}*, in the same gene that restores TNI activity through a L188F change (Prado *et al.* 1995; *i.e.*, males *wupA^{hdp2}*, *wupA^{D3}*; *Dp/Dp* are viable and normal flies). This observation prompted the quantification of the relative viability of genotypes in which one of the extra copies of the region was represented by an *R* duplication. We find that hyperploid genotypes of the type *Dp/R* are, on average, 50% viable with respect to sibling

Dp/+ controls when testing *R1*, *R2*, and *R3* (Table 2). Thus, although the near-lethal effect of two doses of the intact duplication disappears if one of the copies is a reduced version, the fact that the viability is not completely normal suggests that other components of the cluster still produce a deleterious effect if hyperploid. These observations point to a dosage requirement between genes covered by *Dp*, in which TNI seems to play an important role.

Additional evidence for dosage relationships between TNI and the other components of the cluster was obtained from the semidominant lethals. We tested the rescue effects of *R* duplications upon the *SDL* mutations measuring the viability of *SDL/+*; *R/+* females (Table 3). As mentioned above, *SDL/+* females are 10–30% viable, but this near-lethal effect is rescued by *Dp*. We find that *R5* recovers, even better than *Dp*, the semidom-

TABLE 2

Relative viability of aneuploids in the 16F cluster

| Experimental | Viability (%) | Control | | |
|------------------------------|---------------|---------|------------------------------|-------------|
| + | <i>Dp/Dp</i> | 4.5 | + | <i>Dp/+</i> |
| <i>wupA^{hdp2}</i> ; | <i>Dp/Dp</i> | 0.6 | <i>wupA^{hdp2}</i> ; | <i>Dp/+</i> |
| <i>wupA^{hdp3}</i> ; | <i>Dp/Dp</i> | 0 | <i>wupA^{hdp3}</i> ; | <i>Dp/+</i> |
| + | <i>Dp/R1</i> | 44 | + | <i>Dp/+</i> |
| + | <i>Dp/R2</i> | 57 | + | <i>Dp/+</i> |
| + | <i>Dp/R3</i> | 55 | + | <i>Dp/+</i> |

Experimental and control genotypes correspond to sibling males. Viability is expressed as the percentage of experimental vs. control individuals obtained in each cross. Similar values are obtained in females. About 500 individuals were counted in each cross.

TABLE 3

Differential effect of R duplications on *SDL* mutants

| Experimental | Viability (%) | Control |
|------------------------------------|---------------|----------------------------|
| <i>SDL8384/+</i> ; <i>R1/+</i> | 42 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL24597/+</i> ; <i>R1/+</i> | 22 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL8384/+</i> ; <i>R2/+</i> | 0 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL24597/+</i> ; <i>R2/+</i> | 75 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL8384/+</i> ; <i>R3/+</i> | 178 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL24597/+</i> ; <i>R3/+</i> | 63 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL8384/+</i> ; <i>R4-II/+</i> | 0 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL24597/+</i> ; <i>R4-II/+</i> | 46 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL8384/+</i> ; <i>R5/+</i> | 230 | <i>SDL/+</i> ; <i>Dp/+</i> |
| <i>SDL24597/+</i> ; <i>R5/+</i> | 431 | <i>SDL/+</i> ; <i>Dp/+</i> |

Viability is expressed as the percentage of *SDL/+* ; *R/+* adult females obtained with respect to the sibling control *SDL/+* ; *Dp/+*. A minimum of 100 adults of each experimental genotype was scored.

inant lethality of both *SDL* mutations. However, the other *R* fragments show differential effects on viability. In particular, the *R2* and *R4-II* duplications have very different effects, depending on the *SDL* mutation tested. This is another differential trait between the two *SDL* mutations in addition to the body size of the *SDL/+* survivors mentioned above. Considering the chromosomal extent present in each *R* element (Figure 4), there is no obvious correlation with the degree of viability rescue of *SDL/+* genotypes. It is more likely that the nature of the products involved in these aneuploids would be the relevant factor.

Searching for other alternatives: Several possibilities to account for the haplolethal phenomenon have been tested; the results, although negative, deserve to be reported. A role in dosage compensation was an obvious alternative, considering the fact that the *HL* at 16F, as well as four other *HL* loci, is located in the *X* chromosome. Also, the observed lethality of *wupA; Dp/Dp* genotypes (see above) justified the study of *X:A* chromosome dosage compensation (Baker *et al.* 1994). We measured the transcription ratio of two genes in the salivary glands, the *X*-linked *Sgs4* and the autosome III-linked *Sgs3*, in several genotypes from which LIII larvae can be obtained (see materials and methods; Breen and Lucchesi 1986). Neither *wupA^{hdp2}* nor *wupA^{hdp3}* alleles nor aneuploids carrying *Dp(1;3)JC153* seem to modify the *Sgs4:Sgs3* ratio of transcription, indicating that sex-dependent dosage compensation is not affected. Also, lethal mutations at the dosage compensation control genes *msl-1* and *msl-3* (Kuroda *et al.* 1991) neither affected the *DL* condition nor modified their phenotype on a *wupA^{hdp3}* background (*i.e.*, females *DL/+*; *msl/msl* and males *wupA^{hdp3}; msl/msl* are still lethal).

Concerning a potential interaction among the known *HL* loci, we tested in some detail the case of the haplo-triplo-lethal at 83D-E because it is the only other *HL* region that has been subjected to a thorough genetic analysis (Keppy and Denell 1979; Roehrdanz and Lucchesi 1981; Dorer and Christensen 1990; Dorer *et al.* 1995). We find that our *HL⁻* or *DL* genotypes are not modified in their lethality if the 83D-E region is present in either haplo or triplo condition. Studies on the haplo-triplo-lethal locus at 83D-E have shown that it might be functionally related to the 7E3-8A5 region of the *X* chromosome because the *Dp(1;2)sn⁺72d*, named *Isis*, can rescue the triplolethal condition (Dorer *et al.* 1993). We find that *Isis* has no effect in our case. Also, the mutation *Su(Tpl)¹⁰* (Dorer *et al.* 1995) fails to rescue *DL* or *DL/+* genotypes. Our own attempts to generate second site suppressors of *DL* mutations have been unsuccessful so far. Other reported *HL* regions (5A6-13, 7C5-9, 12A1-7, and 22F1-2) were tested for potential rescue of *DL* mutations using their corresponding duplications or deletions; however, no effect was detected. Thus, it appears that the *HL* function at the 16F cluster is a local phenomenon.

The *HL* function at 16F is present in other species of *Drosophila*: We generated hybrids with other species to test if the *HL* at 16F is a peculiarity of *D. melanogaster*. None of the hybrids between *melanogaster* and *simulans*, *mauritiana*, or *teissieri* of the genotypes males *HL⁻*, males *DL*, females *HL⁻/+*, or females *DL/+* survive unless the *Dp(1;3)JC153*-bearing chromosome from *melanogaster* is present in the hybrid genotype. This observation demonstrates that the *HL* function of the 16F cluster has a homologous counterpart in the three species tested.

Although the DNA sequences corresponding to the *DL*, *SDL*, and *RL* domains are known—in fact, the continuous genomic region from *HL-VIII* to *HL-XII* is known—the search in the databanks has not yet shown a revealing homology. In this context, it is unfortunate that most of the sequences available for comparison relate to cDNAs while the *HL* function resides, in all probability, in noncoding sequences.

DISCUSSION

This study reports the genetic analysis on the 16F region, whose haploid condition is lethal. The 100-kb region analyzed contains 14 genes identified either as transcription units and/or complementation groups. We show that the *HL* function can be mutated to a dominant-lethal condition only by means of rearrangements breaking within a 4-kb segment of the troponin I-encoding gene. Considering the 10 most significant rearrangements obtained (Figure 5), their breakpoints serve to identify three DNA domains: *DL*, in which breaks cause a dominant lethal effect; *SDL*, in which rearrangements have a semidominant lethal phenotype; and *RL*, which is represented by the 2-kb deletion *23437*, which yields a recessive lethal effect. The three domains are located in untranslated regions: *DL* and *SDL* within the *wupA* gene and *RL* immediately upstream of this gene. These domains seem to regulate the expression of adjacent genes in a position-specific manner that is hypothesized in Figure 7B. For a normal function, the *DL* domain, at least needs to be located in its native locus, suggesting a long-range *cis* effect. This effect cannot be supplied by the normal *trans* homologous chromosome, even in conjunction with additional transgenic fragments containing a *DL* domain in ectopic positions (Figure 5B).

The cluster of genes: The density of genes per kilobase is clearly not uniform along the chromosomes in any species. Current data on the density of transcription units in the 360 kb of the *Shaker* cluster (*ME*, *V*, and *HL* regions) yield a gene:kilobase ratio of 1:17. This value, however, varies from 1:100 in the *V* region to 1:7 in *HL*. In turn, genes in this cluster appear to be more densely packed than those in the adjacent 190 kb between *V7* and *JC153* (Eberl *et al.* 1992). These data add to those from other genomic regions thoroughly analyzed at the molecular and genetic levels, such as

the 67 kb in 19F, which harbors 12 genes (Maleszka *et al.* 1998), and the ongoing study of 2500 kb in 35BC (Berkeley Drosophila Genome Project). Beyond the generalizing views that estimate average values of gene density and proportion of vital mutations in the whole genome, the emergent picture is a genome composed of regions with very different values for the extent of DNA with transcriptional activity (75% in 19F vs. 50% in 16F) and vital functions (30% in 19F vs. 70% in 16F). Between the whole-genome and the single-gene levels of analysis, it may also be informative to pay attention to an intermediate level: the chromosomal region. The number of clustered genes grouped under the criteria of functional relationships between members is well documented in many species. In *Drosophila*, a paramount example is the dopa decarboxylase cluster of 18 loci that is involved in catecholamine metabolism (Wright 1996). The case of the *Shaker* cluster belongs to the nonsequence-related type, and the functional relationships among the constituents are still defined vaguely. However, *Shaker*-like phenotypes are documented for several genes in the cluster (Ferrús *et al.* 1990). As the genomic analyses have become more systematic, gene clusters with apparent or suspected functional ties are emerging (Bione *et al.* 1993; Hong and Ganetzky 1996). In fact, the possible existence of a functional organization in the chromosome of a higher order than the transcription unit is a subject for which evidence is beginning to accumulate in both prokaryotes (Williamson *et al.* 1993) and eukaryotes (Hiraoka *et al.* 1993; Dernburg *et al.* 1996; Olson *et al.* 1996). This hypothesized level of functional organization requires, among other elements, the existence of regulatory mechanisms of gene expression with a long-range effect. In this context, the three domains described here might represent one of the regulatory mechanisms operating within the 16F cluster.

The haplolethal function: In light of the available data, general interpretation of the HL phenomenon at 16F can be proposed on the basis of regulatory sequences that are needed for the correct expression of TNI and at least two adjacent genes. This putative regulatory activity seems to be very sensitive to the topology of its sequences because a small insertion of 540 bp functionally inactivates the complete set of genes. Interestingly, point mutations apparently fail to produce mutant effects in the DL, SDL, or RL domains, suggesting that the proposed regulation might rely on the high-order structure of the DNA rather than on its primary sequence. We hypothesize that the dominant-lethal effect might result from the combined depletion of functionally related products because of the inactivation of their common regulatory mechanism of expression. Regulatory sequences within, and not upstream of, a transcription unit are a common feature for the TNI-encoding gene in other species (Yutzey *et al.* 1989). The role of high-order DNA structures in the regulation

of a gene or cluster of genes is a less well-defined phenomenon, although experimental evidence is accumulating (Nikovits *et al.* 1990; Rivier and Pillus 1994; Dernburg *et al.* 1996; Arnone and Davidson 1997). However, what makes the case of 16F particularly attractive is the coincidence of a long-range regulatory mechanism and a functional relationship among the regulated genes. This relationship is documented by the lack of complementation in the muscle phenotypes between the viable *wupA* alleles and the lethal mutations for *HL-VI* (Figure 9). This suggests that the *HL-VI* product is likely to interact with TNI in muscles. As for the other components of the cluster, *HL-XII* and *HL-XIII*, the putative relationship is based only on the complementation map of their lethality (Figure 7). At this time, no further phenotypic descriptions are available for these mutations. The extent of the *cis* regulatory effect of the DL, SDL, and RL domains seems to involve only three, possibly four, genes. However, the deleterious effects observed in the partial aneuploids of the region (Tables 2 and 3) suggest that other members might have functional interactions as well. Indeed, this has been proven in the only case that has been studied in detail, the Ca²⁺-binding protein Frequentin and the K⁺ channels of *Shaker*, where the former is a negative modulator of the latter (Bourret-Poulain *et al.* 1994). In the same context, it should be noticed that the *HL-VI* mutation 1614 shows a *Shaker*-like dominant alteration of action potentials (see Figure 8 in Ferrús *et al.* 1990). The three domains seem to effect their role in the same *cis* direction, toward the centromere; however, they differ in their extent (Figure 7B). *RL* does not seem to affect the expression of *wupA*, while *DL* and *SDL* do not seem to affect the putative *HL-XIII*. At this time, the phenotypic differences between the mutations in *DL* and *SDL* cannot be interpreted on the basis of the differential extent of their realm of action. The fact that the *SDL*/+ genotype either dies early in development or survives with normal morphology and behavior into adulthood suggests that a critical event takes place early in development, which leads to a permanent state of ON or OFF expression for one or several genes. The types of events described here are likely to be based on specific forms of chromatin structure. Consequently, they are similar to the phenomena of position effect variegation (Reuter and Spierer 1992; Judd 1995) and *cis*-regulation (Wilson *et al.* 1990) in general. In this context, there is evidence for the functional relevance of chromatin structure through specific sequences in the normal expression of troponin I-encoding genes in vertebrates (Nikovits *et al.* 1990; Lin *et al.* 1991; Yutzey and Konieczny 1992).

It is not possible to venture an interpretation of the HL effect in other regions of the genome, and the attempts carried out in this study indicate that the various HL regions are functionally independent of each other. However, it is still quite striking that, as first

noticed by Homyk and Emerson (1988), most HL regions include a gene that encodes cytoskeletal proteins (Table 1). The rationale of this proposal was based on the qualitative and quantitative importance of this type of protein in all cell types (Kares *et al.* 1991; Stossel 1993; Young *et al.* 1993). At 16F, this is not just a coincidence, but a relevant feature that is represented by troponin I. However, other proteins in addition to TNI contribute to the haplolethality. More relevant than the presence of a cytoskeletal protein is possibly the fact that the *DL* mutations result only from DNA rearrangements. This observation holds true in 16F and in 83D-E (Roehrdanz and Lucchesi 1980), the only two loci in which *DLs* have been searched for. Further studies in other HL regions will show how general this observation is. Still unresolved is the striking feature of the abundance of HL regions in the *X* chromosome. It could have been reasoned that haplolethality would have been the result of an inefficient dosage compensation in recently transposed genes from the autosomes to a sexually dosage-compensated chromosome. However, the presence of a homologous HL function in the three species tested makes this possibility unlikely, and the expression of *wupA* in *D. melanogaster* has certainly been sexually compensated properly. The alternative that *wupA* mutations have a direct role in dosage compensation is also unsupported by the available data, even though the *DL* mutants could not be tested for dosage-compensation defects. Finally, it should be pointed out that haplolethals have been discovered in *D. melanogaster* because aneuploid analysis is feasible, and, furthermore, the isolation of *DL* mutants is allowed, provided that a suitable duplication is at hand. None of these technical conditions are available in any other species. Thus, the HL phenomenon might be a more abundant feature than what is presently known.

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