hobo Induced Rearrangements in the yellow Locus Influence the Insulation Effect of the gypsy su(Hw)-Binding Region in Drosophila melanogaster

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ABSTRACT

The su(Hw) protein is responsible for the insulation mediated by the su(Hw)-binding region present in the gypsy retrotransposon. In the y mutation, su(Hw) protein partially inhibits yellow transcription by repressing the function of transcriptional enhancers located distally from the yellow promoter with respect to gypsy. y mutation derivatives have been induced by the insertion of two hobo copies on the both sides of gypsy: into the yellow intron and into the 5′ regulatory region upstream of the wing and body enhancers. The hobo elements have the same structure and orientation, opposite to the direction of yellow transcription. In the sequence context, where two copies of hobo are separated by the su(Hw)-binding region, hobo-dependent rearrangements are frequently associated with duplications of the region between the hobo elements. Duplication of the su(Hw)-binding region strongly inhibits the insulation of the yellow promoter separated from the body and wing enhancers by gypsy. These results provide a better insight into mechanisms by which the su(Hw)-binding region affects the enhancer function.

InsTATIONS of gypsy (mdg4) retrotransposons into various Drosophila melanogaster genes result in mutations with phenotypes that can be reversed by second site mutations in the suppressor of hairy-wing [su(Hw)] gene (Modicheletal al. 1983). This effect has been extensively studied by using the yellow (y) gene (Corces and Geyer 1991). The gypsy-induced y allele displays a tissue-specific mutant phenotype characterized by the loss of pigmentation in the wings and in the body cuticle, whereas all other tissues of the larvae and adult flies show the wild-type coloration (Nash and Yarkin 1974). In this mutation, gypsy was inserted at −700 bp from the transcription start site of the yellow gene. The enhancers controlling yellow expression in the wings and in the body cuticle are located upstream of the gypsy insertion site (Geyer et al. 1986; Parkhurst and Corces 1986; Geyer and Corces 1987; Martin et al. 1989). The region of gypsy responsible for its mutagenic effect is the binding site for the su(Hw) protein (Parkhurst et al. 1988; Spana et al. 1988; Mazo et al. 1989; Dorsett 1990; Spana and Corces 1990). Thus, it has properties characteristic of a chromatin insulator: only enhancers located distally from the promoter are affected (Corces and Geyer 1991; Holdridge and Dorsett 1991; Jack et al. 1991; Geyer and Corces 1992; Roseman et al. 1993; Cai and Levine 1995; Scott and Geyer 1995). The second gene that affects gypsy-induced phenotypes, modifier of mdg4 [mod(mdg4)], encodes a protein that interacts with su(Hw). Mutations in mod(mdg4) enhance the phenotype of the y′ by inactivating yellow transcription (Geyer and Gerasimova 1989; Geyer and Corces 1995), either due to changes in the chromatin structure that interferes with the function of all enhancers of the yellow gene (Gerasimova et al. 1995; Gerasimova and Corces 1996) or by direct inhibition of the yellow promoter (Geyer and Kozychina 1996).

In this article, we describe the genetic instability induced by hobo transposable elements in derivatives of the y′ mutation. hobo is a small transposon (3 kb in size) with short inverted repeats (Streck et al. 1986). The largest hobo element encodes a transposase that is specific for the members of the hobo family (Blackman et al. 1989; Calvi et al. 1991). The first derivative of the y′ allele was induced by the insertion of two hobo elements in the intron of the yellow gene and in the 5′ regulatory region downstream to the body and wing enhancers. Both hobo elements had the same direction and identical restriction maps. In contrast to previous observations (Calvi et al. 1991; Ho et al. 1993; Sheen et al. 1993), duplications of the region between hobo elements occurred with a high frequency. The duplications included the regulatory region of the yellow gene and gypsy sequences. Flies with such duplications showed the wild-type level of pigmentation of the body and wings, which seemed to be due to the normal expression
of the yellow gene controlled by the yellow transcriptional enhancers, although they remained flanked by the su(Hw)-binding region.

MATERIALS AND METHODS

**Stocks:** Flies were cultured at 25°C in standard Drosophila wheatmeal, yeast, sugar, and agar medium. All crosses were performed in standard glass vials with 5-10 males and 10-15 females per vial. Additional information about the genetic markers can be found in Lindsey and Zimm (1992).

The following strains were synthesized in the previous work (Georgiev and Kozyczna 1996): XX/Y; Xa/D, XX/Y; su(Hw)/Xa, XX/Y; su(Hw)/Xa, XX/Y; mod(md132/2 mod(md54/13), where XX is an abbreviation for C(1)RM, y; X is an abbreviation for the translocation T(2;3) ap*/ap*.

**Genetic crosses:** The ysc^w1 allele strain contains about 20 hobo copies. The C(1)RM, y strain has no hobo elements. Crosses of ysc^w1 males with C(1)RM,y females activate the transposition of hobo. To study hobo-mediated rearrangements in the y alleles, dysgenic ysc^w1/y females (y - hobo-induced y allele) were individually crossed to 6-8 C(1)RM,y females. The males with a new y phenotype were mated to C(1)RM,y females, and the phenotype was examined in the next generation. Only the similar events obtained from independent males were referred to as independent events. The stocks with new y alleles were established, but in general they retained some level of instability, and the males with the new y phenotype appeared with a low frequency, \( \sim 1 \times 10^{-4} \).

The phenotypic analysis was performed at 25°C in 3-5-day-old males. The results were compared with those obtained in control flies with a known phenotype (Georgiev et al. 1992). The degree to which the y alleles differed from the wild type was determined visually. The wild-type expression was estimated at 5 points, whereas the absence of yellow expression was indicated by 0.

To study the influence of the su(Hw)/su(Hw) heterozygote or the mod(md132/2 mod(md54/13) homozygote on the expression of the y alleles, the following crosses were carried out. Males with a y allele to be tested were crossed to C(1)RM,y females carrying the Drop D mutation as a dominant marker. F1; y; D + males were crossed to C(1)RM,y; su(Hw)/Xa or C(1)RM,y; mod(md132/2 mod(md54/13) females. F1; y; D/su(Hw) or D/su(Hw) males were crossed to C(1)RM,y; su(Hw)/Xa or C(1)RM,y; mod(md132/2 mod(md54/13) females. Analysis of the phenotype of y; su(Hw)/y; su(Hw) or y; mod(md132/2 mod(md54/13) males was performed at 25°C in the F0 or F1 generation. The results were compared with those obtained in control flies.

**Molecular methods:** For Southern blot hybridization, DNA from adult flies was isolated using the protocol described by Ashburner (1989). Treatment of DNA with restriction endonucleases, blotting, fixation, and hybridization with radioactive probes prepared by random primer extension was performed as described in the protocols for the Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and in the laboratory manual (Sambrook et al. 1989). Phage with cloned regions of the yellow locus were obtained from M. Marlow (Campuzano et al. 1985) and V. Corces (Geyer et al. 1986). The probes were made from gel-isolated fragments after an appropriate restriction digestion of plasmid subclones.

For Northern blot hybridization, total RNA was extracted at the pupal stages by using the sodium dodecyl sulfate (SDS)-phenol technique (Spradling and Mahowald 1979). The samples were homogenized in 10 ml of 10 mm Tris-HCl (pH 7.4), 100 mm NaCl, 1 mm EDTA, 0.5% SDS, and the homogenate was extracted several times with phenol-chloroform with subsequent chloroform extraction. Poly(A)^+ RNA was then isolated by chromatography on oligo(dT)-cellulose and fractionated by electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Keene, NH), and incubated with ^32P-labeled probes. The DNA fragment used as a hybridization probe to detect the yellow transcript was obtained by digestion of the cDNA clone of the yellow gene with HindIII and BglII restriction endonucleases. The yellow cDNA clone was obtained from P. Geyer.

Genomic DNA libraries were constructed using DNA partially digested with Sau3A. The digested DNA was ligated in the Xgen11/BamHI phage vector (Promega, Madison, WI). The recombinant DNA was packaged in vitro using a packaging extract from Promega, and the phage particles were plated using the Escherichia coli strain LE392 at a density of 3000 pfu/plate. The plaques were blotted onto Hybond-N nylon membranes according to the supplier protocol (Amersham). These membranes were hybridized with ^32P-labeled DNA probes to select the desired plaques; 30,000-40,000 plaques from each recombinant DNA library were screened. Positive plaques were picked up from the plates and rescreened to obtain pure clones.

In other cases, DNA samples were restricted with BamHI endonuclease and subjected to agarose gel electrophoresis. Bands of corresponding size were cut from the gel, and DNA was extracted by electrophoresion. After that, the DNA was ligated to the arms of the Xgen11/BamHI phage vector (Promega). Subcloning and purification of the plasmid DNA and mapping of restriction sites were performed by standard techniques (Sambrook et al. 1989).

Genomic DNAs were subjected to PCR to amplify sequences from the y allele (Saiki et al. 1985; Mullis and Faloona 1987). The primers used in DNA amplification were as follows: from the bo hobo element, 5'GACTGACTACCTAGGACC3' (h1, 313-293 according to the map of the hobo element described by Streck et al. (1986); from the y locus, 5'GAATGTTGGTGTGCTGTTG3' (y1, 1181-1159 (Geyer et al. 1986)) and 5'TCTGTGGAGCTTGGGCAATAC3' (y2, 2899-2877); from the gypsy mobile element, 5'CAACCTTGCAAGGACTCTTAG3' (g1, 2674-2696 (Marlov et al. 1986)). The products of amplification were fractionated by electrophoresis in 1-2% agarose gels in Tris-acetate (TAE) buffer.

DNA sequencing was performed according to the dideoxy chain-termination methodology (Sanger et al. 1977). The PCR product was directly sequenced using a Sequenase II sequencing kit for PCR product (Amersham) according to the manufacturer's instructions.

RESULTS

The original hobo-induced y allele, y^h1, contains the su(Hw)-binding region surrounded by two hobo elements: The original y^h1 allele spontaneously appeared in the ysc^w1 strain. In the parental y allele (the yellow color of the body cuticle and wing blade), yellow transcription in the body and wings was blocked by the su(Hw)-binding region of gypsy. By contrast, y^h1 flies displayed a weak pigmentation of the wings and, in addition, the mutant color of bristles on the notum and legs (Table 1).

To understand the molecular basis, the y^h1 allele was cloned. A recombinant DNA library was probed with the Sall-BglII and HindIII-BamHI fragments from the yellow locus. Three recombinant phages hybridizing with
hobo Duplications Affect Insulation

TABLE 1
Phenotypes of hobo-induced yellow alleles and the effect of the su(Hw) mutation

<table>
<thead>
<tr>
<th>y alleles</th>
<th>Body</th>
<th>Wings</th>
<th>Th</th>
<th>L</th>
<th>W</th>
<th>Ab</th>
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<tr>
<td>y²</td>
<td>1(5)</td>
<td>1(5)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>y⁶h</td>
<td>1(5)</td>
<td>2(5)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>y⁹</td>
<td>5(5)</td>
<td>5(5)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>²h115</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>y²h</td>
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<td>3(5)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>y²h</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>²h115</td>
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<td>1(3)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>y²h²</td>
<td>1(1)</td>
<td>1(2)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>²h²</td>
<td>1(1)</td>
<td>1(2)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>²h²</td>
<td>1(2)</td>
<td>1(3)</td>
<td>2(5)</td>
<td>3(5)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>²h²</td>
<td>1(2)</td>
<td>1(4)</td>
<td>2(5)</td>
<td>3(5)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>²h²</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Bristles are subdivided into thoracic (Th), leg (L), wing (W), and abdominal (Ab). The number in parentheses shows the effect of the su(hw)/su(hw) mutations combination. For determination of the yellow phenotype, the levels of pigmentation in different tissues of adult flies were estimated visually by comparison with the changed fragments and their detailed mapping.

Hobo Duplications are frequently associated with duplications of the region between two hobo elements: In the F₂ generation from dysgenic crosses (see above), two main classes of mutant derivatives were obtained from the y⁹ strain (Tables 1 and 2): y⁹ (complete inactivation of the yellow gene) and y⁹ (normal pigmentation of the body and wings but a mutant phenotype in the notum and leg bristles).

Two examined y⁹ alleles had a deletion of yellow and gypsy sequences located between the hobo elements (Figure 2A). The other three y⁹ alleles were induced by deletions extending from hobo-1 to the yellow region located to the left or to the right of the hobo insertion (data not shown). As a result, yellow expression was completely inactivated.

DNAs from six independent y⁹ alleles were probed with the fragments of the yellow gene. All bands characteristic of y⁹ DNA were detected. At the same time, additional hybridizing bands appeared that were the same in all DNA samples (Figure 2, B and C). It was suggested that the y⁹ alleles were associated with the duplication of some parts of the yellow gene. We cloned DNA fragments of y⁹ corresponding to the two BsmI bands obtained in the course of electrophoresis, which hybridized to the yellow probe. Detailed restriction maps of the cloned DNAs are shown in Figure 3. The y⁹ allele was derived by the duplication of the region between two hobo elements. All repeated elements in y⁹ and other mutations with the duplication are numerated in the yellow-proximal to the yellow-distal direction (hobo-1, 2, and 3, gypsy-1 and 2, etc.)
The DNA of y^m^7 flies that had been restricted with BamHI or BglII differed from other y^m^ alleles in Southern blot analysis (Figure 2, C and D). The y^m^7 allele had a 6-kb deletion that occupied the region extending from the hobo-3 element and partially included gypsy-2, which left the su(Hw)-binding region of the latter unchanged (Figure 3). PCR cloning of the deletion breakpoints showed that the sequences between the 5' end of the hobo-3 element and 3428 bp in the gypsy sequence were deleted (according to the gypsy map presented by Marler et al. 1986).

It may be concluded that the duplication of the hobo-flanked region is the main mutagenic event in the system.

**Loss of insulation in the y^m^ alleles:** As has been shown above for y^m^ alleles, the duplication of hobo-2, body and wing enhancers, gypsy, and yellow promoters led to the restoration of yellow expression. The su(Hw)^/+ su(Hw)^/ heterozygote did not visually change the phenotype of y^m^2, y^m^3, y^m^4 and y^m^9 flies (Table 1). Thus, the duplication made it possible to somehow overcome the su(Hw)^/-dependent insulation of the yellow gene.

The y^m^7 flies contain a deletion of distal yellow enhancers. Thus, in the presence of two su(Hw)-binding regions and two promoters, one pair of yellow enhancers, that is, the proximal body and wing enhancers, restored yellow transcription. The result could be explained either by the loss of insulation in a particular sequence context or by initiation of transcription from the distal promoter, yellow promoter 2, by the proximal enhancers not isolated from the latter by the su(Hw)-binding region.

To check whether the yellow promoter was properly activated in the system, the size and time of accumulation of yellow mRNA at the pupal stage were measured by Northern blot analysis (Figure 4). The RNAs isolated at three pupal stages from the y^m^1 and Oregon strains had the same size, level, and time of expression, suggesting that the yellow gene in the mutant was transcribed from the normal promoter and was activated by its native enhancer elements.

Finally, we obtained one derivative allele, y^m^h^3^2, as a result of rearrangement between the hobo elements in the yellow and neighboring achaete-scute complex. The body and wing pigmentation of the y^m^h^3^2 flies was close to wild type (Table 1). The origin of the mutation is quite different from mutations of the same class described above (M. Gause and P. Georgiev, unpublished results).

Briefly, the mutation was found to be induced by an additional inversion of the region between hobo-2 and hobo located in the scute gene close to the gypsy su(Hw)-binding region (Figure 5). As a result, the body enhancer and part of the wing blade enhancer were flanked by two gypsy su(Hw)-binding regions and isolated from the yellow promoter 1. In this case, transcription of the yellow gene could start only from promoter 1, which is isolated from the enhancers by the su(Hw)-binding region. The decrease of pigmentation in the y^m^h^3^2 flies may be explained by a deletion of the part of
the wing enhancer (Figure 5). This result confirms the suggestion that the activation of yellow transcription in the body and wings really depends on the loss of insulation in the presence of two gypsy elements.

**Genetic and molecular analysis of y<sup>th</sup> derivatives.** To obtain y<sup>th</sup> derivative mutations, males from three independent strains, y<sup>y1h</sup>, y<sup>y2</sup>, and y<sup>y9</sup>, were crossed to C(1)RM,yf females (Table 2). New alleles fell into four phenotypic classes: y<sup>y1h</sup> (complete inactivation of the yellow gene); y<sup>y2</sup> (phenotype as in the parental y<sup>y1h</sup> allele); y<sup>y9</sup> (yellow notum and leg bristles and an intermediate level of bodyand wing pigmentation); and y<sup>y2h</sup> (the yellow color of the body and wings as seen in y<sup>y1h</sup>). DNA's from eleven randomly selected y<sup>y1h</sup> alleles did not hybridize to the probes from the yellow gene, indicating that these mutations represent deletions of se-

**TABLE 2**

<table>
<thead>
<tr>
<th>Original y allele</th>
<th>Total number of flies scored</th>
<th>Main derivative alleles and a number of mutations obtained</th>
<th>Total frequency of mutagenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>y&lt;sup&gt;y1h&lt;/sup&gt;</td>
<td>2840</td>
<td>y&lt;sup&gt;y1h&lt;/sup&gt; 9 y&lt;sup&gt;y2&lt;/sup&gt; — — 7</td>
<td>5.6 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>y&lt;sup&gt;y1h&lt;/sup&gt;, y&lt;sup&gt;y2&lt;/sup&gt;, y&lt;sup&gt;y9&lt;/sup&gt;</td>
<td>8400</td>
<td>— 8 21 — 7 15</td>
<td>6.1 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>y&lt;sup&gt;y1h&lt;/sup&gt;, y&lt;sup&gt;y11&lt;/sup&gt;</td>
<td>1620</td>
<td>5 21 7 15 6</td>
<td>3.8 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The figures indicate the number of independent events, that is, the number of similar events obtained from different dysgenic F<sub>1</sub> males. Total frequency of mutagenesis means the ratio of the number of independent events to the total number of scored flies.
quences between hobo-1 and hobo-3 (Figure 6). Ten derivative y^h alleles had the same structure as the original y^h allele; that is, they were also induced by a recombination-mediated deletion of the sequences between hobo-1 and hobo-2, between hobo-2 and hobo-3 elements, or between gypsy-1 and gypsy-2 (Figure 6). Two y^h and three y^h alleles appeared to result from complex inversions and additional duplications (data not shown). These alleles were not studied further.

Two other classes of mutations, y^h and y^h, were studied in more detail.

**The nature of y^h mutations:** y^h flies had yellow notum and leg bristles but an intermediate level of body and wing pigmentation (Table 1). Four extensively studied y^h DNAs restricted with BamHI gave just one 24-kb band hybridizing to yellow gene probes (Figure 6). The 24-kb BamHI fragment of y^h DNA was cloned. A detailed restriction map of the cloned y^h is shown in Figure 7A. The y^h mutation was caused by recombination between 5'-LTR of the gypsy-2 and 3'-LTR of the gypsy-1 and, as a result, the hobo-2 and yellow sequences located between the gypsy elements were deleted. According to Southern blot analysis, all y^h alleles had the same structure, that is, they possessed two gypsy elements, lacking the intervening sequences. The su(Hw)^2/su(Hw)v heterozygote suppressed the mutant phenotype of y^h alleles (Table 1). This suggests that the su(Hw)-binding region partially but not completely blocks the body and wing enhancers in these alleles.

To study the regulatory region responsible for the yellow activation in y^h flies, we obtained the derivatives of the y^h and y^h alleles (Table 2). The major class of flies with a new mutation phenotype was y^h. The mutant flies had no pigmentation of the body cuticle and the wing blade (Table 1).

Four mutant alleles were subjected to a molecular analysis: y^h, y^h, y^h, and y^h. Southern blot analysis showed deletions of 5 kb (y^h, y^h), 7 kb (y^h) and 8 kb (y^h) in the region flanking the hobo-2 element (Figure 7B). The deleted regions included the body and wing enhancers and a portion of the gypsy sequences (Figure 7A). In combination with the su(Hw)^2/su(Hw)v heterozygote, y^h and y^h alleles exhibited only a slightly enhanced wing pigmentation. This result suggests that the body and wing blade enhancers can par-

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**Figure 3.—The structure of the y^h, y^h, and y^h alleles.** The thin lines show the deletions in the y^h and y^h alleles. Other designations are as in Figure 1. The breakpoints of deletion in the y^h allele were cloned by PCR between the primers in the hobo element (h1) and in the gypsy (g1). yp1, yellow promoter 1; yp2, yellow promoter 2.

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**Figure 4.—Analysis of yellow transcripts in the mutant strains.** Northern blot hybridization was performed with RNA isolated from y^h(1, 4, 8), y^h(3, 6, 9), and control Oregon strains. Northern blot hybridization was performed with RNA isolated from y^h(1, 4, 8), y^h(3, 6, 9), and control Oregon flies(2, 5, 7). Poly(A)^+ RNAs were isolated from 0–24-hr pupae (1–3), 48–72-hr pupae (4–6), and 72–96-hr pupae (7–9). **Figure 4.—Analysis of yellow transcripts in the mutant strains.** Northern blot hybridization was performed with RNA isolated from y^h(1, 4, 8), y^h(3, 6, 9), and control Oregon flies(2, 5, 7). Poly(A)^+ RNAs were isolated from 0–24-hr pupae (1–3), 48–72-hr pupae (4–6), and 72–96-hr pupae (7–9). **Figure 4.—Analysis of yellow transcripts in the mutant strains.** Northern blot hybridization was performed with RNA isolated from y^h(1, 4, 8), y^h(3, 6, 9), and control Oregon flies(2, 5, 7). Poly(A)^+ RNAs were isolated from 0–24-hr pupae (1–3), 48–72-hr pupae (4–6), and 72–96-hr pupae (7–9).
tially activate yellow expression in \(y^h\) flies when separated from the promoter by two su(Hw)-binding regions.

**The nature of \(y^h\) mutations:** \(y^h\) flies had the same level of wing and body pigmentation as \(y^\ell\) flies (Table 1). According to Southern blot analysis, the \(y^{212}\), \(y^{215}\), \(y^{216}\), \(y^{218}\), and \(y^{219}\) alleles had a deletion of the duplication and of the adjacent yellow sequences (Figure 7). There were some minor phenotypic differences between different alleles correlating with the size of the deletion.

The color of bristles in \(y^{212}\) and \(y^{215}\) flies is similar to that of the \(y^{11}\) allele. In the \(y^{215}\) allele, only sequences between hobo-2 and the proximal Bgl II site were deleted (about 600 bp). PCR cloning and sequencing showed that the sequences were deleted between \(-2463\) and \(-1953\) positions relative to the transcription start site of the yellow gene. The mutant \(y\) phenotype of the \(y^{215}\) allele was suppressed in the body and partially in the wings in the su(Hw)\(^{1}/\) su(Hw)\(^{\ell}\) heterozygote (Table 1). This was expected because the previously defined body enhancer (from \(-1963\) bp to \(-1266\) bp) was present in its entirety, together with a portion of the wing blade enhancer (\(-2873\) bp to \(-2463\) bp).

In the \(y^{2112}\) allele Southern blot hybridization showed a 2-kb deletion (Figures 2D and 8B). Thus, sequences from \(-2463\) to \(-700\), between hobo-2 and gypsy 3'-LTR, were deleted. In the \(y^{2112}\) allele only a part of the wing blade enhancer between \(-2873\) and \(-2463\) was present. However, the su(Hw)\(^{1}/\) su(Hw)\(^{\ell}\) heterozygote in combination with \(y^{2112}\) allele still partially increased the pigmentation of the wings (Table 1) and the last segment of the abdomen in \(y^{2112}\) flies (data not shown).

The other three alleles, \(y^{2116}\), \(y^{2125}\), and \(y^{2129}\), exhibited a more extensive pigmentation of the notum and leg bristles. The \(y^{2116}\) allele had a deletion of about 5 kb that spread from hobo-2 into the gypsy body sequences. The \(y^{2125}\) allele had the largest deletion, from hobo-2 up to the border of the su(Hw)-binding region. PCR cloning and sequencing showed that only \(1178\) bp from the 5' end of gypsy were present in the \(y^{2129}\) allele, including the su(Hw)-binding region. The su(Hw)\(^{1}/\) su(Hw)\(^{\ell}\) heterozygote in combination with \(y^{2116}\) and \(y^{2125}\)
alleles significantly increased the pigmentation of the body, wings, and the tip of the abdomen and also completely suppressed the mutant y phenotype in bristles. This means, first, that about half of the wing enhancer can partially support yellow expression, not only in the wings but also in the body and in the tip of the abdomen. Second, both the gypsy body sequences and the su(Hw)-binding region participate in hobo-dependent repression of yellow transcription in the bristles.

This conclusion was supported by data on the structure of y2h29, which had the same phenotype as y (Table 1). The Southern blot analysis, PCR cloning, and sequencing showed that the y2h29 allele had a deletion extending from hobo-2 to the gypsy 5' LTR, thus removing the su(Hw)-binding region. In addition, an inversion between hobo-2 and another hobo located in an unidentified region of the genome removed the last part of the wing blade enhancer, from -2873 to -2463. Thus, in the absence of gypsy, the mutant bristle phenotype was completely reverted. Obviously, the su(Hw)'/su(Hw) tether heterozygote did not increase the pigmentation of y2h29 flies.

The presented results suggest that the body and wing enhancers have a modular organization and partially overlapping functions, in contrast to previous data (Geyer and Corces 1987; Martin et al. 1989). In addition, we show that the region of the wing enhancer located distally from -2463 is responsible for yellow activation not only in the wings but also in the tip of the abdomen.

The effect of the mod(mdg4)1u1 mutation on the phenotype of the hobo-induced y alleles: The mod(mdg4) protein is a second component involved in insulation by the su(Hw)-binding region. Previously, the mod(mdg4)1u1 mutation was shown to repress yellow expression in y mutants (Table 3). These flies had yellow color of the body cuticle, wing blades, and all kinds of bristles, including both wing and abdominal ones. As has been shown above, the insertion of a hobo mobile element, and especially the duplication of gypsy and yellow sequences, strongly influence the insulation properties of the su(Hw)-binding region.

To achieve a better understanding of the role of the mod(mdg4) protein, we studied the effect of the mod(mdg4)1u1 mutation on the phenotypes of the hobo-induced alleles; the flies with all tested y alleles in combination with mod(mdg4)1u1 displayed the yellow color of the bristles (Table 3). In y flies, the mod(mdg4)1u1 mutation...
The structure of the \( y^2h \) alleles, derivatives of \( y^n \). (A) Structure of the \( y^n \) alleles. The arrows show the size of deletions. All other designations are as in Figure 1. The breakpoints of deletion in the \( y^{2h15} \) and \( y^{2h25} \) alleles were cloned by PCR between the primers in the \( hobo \) element (h1) and in the yellow gene (y1 and y2). (B) Southern blot analysis of the \( y^n \) alleles. DNAs from \( y^{2h16} \) (1,14), \( y^{2h2} \) (2,6,15), \( y^{2h3} \) (3), \( y^{2h1} \) (4,7,13), \( y^{2h6} \) (5,10,12), \( y^{2h2} \) (8), \( y^{2h15} \) (11), and \( y^{2h2} \) (9) were digested with BamHI (1-5) or NcoI (6-11) or EcoRI (12-15). The blots were probed with the \( HindIII-BamHI \) fragment from the yellow locus.

Figure 8.

only partially decreased the level of body and wing blade pigmentation. Unexpectedly, in \( y^n \) flies, the mod (mdg4)1u1 mutation failed to influence the normal wing and body pigmentation, although the pigmentation of bristles was reduced in all cases.

It was shown previously that the tip of the abdomen in \( y^n \) mod(mdg4)1u1 males had darker pigmented dots on the cuticle against a background of the mutant-colored cuticle characteristic of \( y^2 \) flies (Gerassimova et al. 1995). \( y^n \) or \( y^n \) mod(mdg4)1u1 males exhibited the same variegation in the abdomen tip pigmentation (data not shown). However, mod(mdg4)1u1 failed to change the pigmentation in the tip of the abdomen in males with \( y^n \) alleles. Thus, in the presence of the mod(mdg4)1u1 mutation, the body and wing enhancers are important for the variegated phenotype of pigmentation in the abdomen tip.

**DISCUSSION**

**hobo-Mediated rearrangements in the presence of the su(Hw)-binding region are frequently associated with duplications:** Previous genetic and molecular studies showed that hobo elements were capable of mediating frequent chromosome rearrangements (Blackman et al. 1987; Hatzopoulos et al. 1987; Johnson-Schlitz and Lim 1987; Yannopoulos et al. 1987; Lim 1988; Ho et al. 1993). It was suggested by Lim (1988) that homologous intrachromosomal recombination between hobo elements was responsible for such rearrangements. The hobo-mediated rearrangements were largely confined to individual chromosome arms (Laverty and Lim 1982; Blackman et al. 1987; Johnson-Schlitz and Lim 1987; Lim 1988), suggesting that they were produced mainly as the result of intramolecular recombination. They were dependent on the orientation of hobo (Lim 1988; Lim and Simmons 1994; Eggleston et al. 1996). When preexisting elements were in the same orientation in a chromosome, the outcome was a deletion of the intervening material and the presence of a single hobo at the deletion breakpoint. Lim (1988) and Lim and Simmons (1994) proposed a model in which hobo elements induced chromosome restructuring via homologous pairing and recombination between the elements at ectopic sites in the genome.

In the \( y^{h1} \) allele, both hobo elements are inserted in
TABLE 3

Influence of the mod(mdg4) yh1 mutation on the hobo-induced yellow alleles

<table>
<thead>
<tr>
<th>y alleles</th>
<th>Body</th>
<th>Wings</th>
<th>Th</th>
<th>L</th>
<th>W</th>
<th>Ab</th>
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</tr>
<tr>
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<td>2(0)</td>
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<td>2(0)</td>
<td>5(1)</td>
<td>5(1)</td>
</tr>
<tr>
<td>yh</td>
<td>5</td>
<td>5</td>
<td>1(0)</td>
<td>2(0)</td>
<td>5(1)</td>
<td>5(2)</td>
</tr>
<tr>
<td>yh</td>
<td>3(1)</td>
<td>3(2)</td>
<td>1(0)</td>
<td>2(0)</td>
<td>5(1)</td>
<td>5(1)</td>
</tr>
<tr>
<td>yh</td>
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<td>1(0)</td>
<td>1(0)</td>
<td>2(0)</td>
<td>5(0)</td>
<td>5(0)</td>
</tr>
<tr>
<td>yh16, yh25</td>
<td>1(0)</td>
<td>1(0)</td>
<td>1(0)</td>
<td>2(0)</td>
<td>5(0)</td>
<td>5(0)</td>
</tr>
</tbody>
</table>

Designations are as in Table 1. The numbers in parentheses show the effect of the homozygous mod(mdg4) yh1 mutation on the phenotype of the y alleles.

The role of hobo in yellow expression: hobo insertions into the y locus led to the reduction of notum and leg bristle pigmentation. This may be explained by the fact that one hobo element inserted into the intron of the yellow gene, exactly in the region of the bristle enhancer. No alleles associated with the excision of the hobo element were obtained, although deletions of gypsy sequences partially restored the bristle pigmentation. The su(H w) mutations suppressed the mutant bristle phenotype, particularly those of yellow alleles that had a deletion of some parts of gypsy, but not of su(H w)-binding region. Recently, we have also found that gypsy sequences other than the su(H w)-binding region can influence the expression of the yellow gene (P. Georgiev and T. Belenkaya, unpublished results). Thus, gypsy sequences, su(H w)-binding region and the hobo insertion have additive negative effects on yellow expression in bristles.

A new insight in the enhancer/promoter insulation by the su(H w)-binding region: An insulator is a sequence that prevents activation or repression from extending across it to the promoter. Only few direct examples of insulators have been reported. Kelum and Schedl (1991) showed that the hsp70 locus of Drosophila melanogaster was bordered by two sequences, scs and scs', that protected it from the effects of neighboring chromatin. The core 0.5-kb scs' element binds the boundary-element-associated factor, which is responsible for the insulation function (Zhao et al. 1995). Chung et al. (1993) identified a component of the β-globin gene cluster that prevented the action of the enhancer on the promoter. The su(H w)-binding region is the most extensively studied insulator element that exhibits remarkable directionality (Corces and Geyer 1991; Holdridge and Dorsett 1991; Jack et al. 1991; Geyer and Corces 1992; Roseman et al. 1993; Cai and Levine 1995; Scott and Geyer 1995).

Our results show that two hobo mobile elements inserted at the yellow intron and the 5' regulatory region (y h alleles) allow the body and wing enhancers to partially overcome the insulation effect of the su(H w)-binding region and to slightly activate the yellow promoter. A possible explanation is that ectopic pairing between hobo elements may interfere with su(H w) insulation. A role for pairing between the homologous elements in the partial suppression of su(H w)-mediated insulation is supported by our analysis of yh alleles. In these alleles, the yellow promoter is isolated from body and wing blade enhancers by two copies of gypsy. Previous studies suggested that the greater the number of su(H w)-binding sites, the more effective the insulation (Hoover et al. 1992; Smith and Corces 1992; Hoever et al. 1992). However, the duplication of the su(H w)-binding region in the yh has an opposite effect: the body and wing blade enhancers partially activate the yellow promoter. Thus, it is possible that the pairing between gypsy sequences or interaction between su(H w)-binding regions partially neutralize the enhancer-blocking effect.

Duplication of gypsy and the yellow sequences located between two hobo elements in the y h alleles restored the insulated yellow expression. This phenomenon may be explained in several different ways. One possibility is that the duplicated yellow promoter in the y h alleles is not isolated by the su(H w)-binding region from the...
wing and body enhancers located downstream. The yellow transcription may pass the hobo, gypsy, and yellow gene sequences. In this case, mRNA of normal size may arise from splicing between the first distal exon located between gypsy-2 and hobo-2 and the second exon of the yellow gene. However, it is difficult to explain in this way the absence of other mRNAs expected to appear in the course of alternative splicing, for example, between the proximal first exon and the second exon of the yellow gene.

Of particular importance are the data on the yh32 allele obtained as a result of inversion between hobo elements located in the yellow and scute loci (M. Gause and P. Georgiev, unpublished results). In this allele the yellow expression is activated by the wing blade and body enhancers located between two gypsy elements in the absence of the second noninsulated promoter. This supports an alternative explanation for the phenotype of yh alleles: that ectopic intrachromosomal pairing between two gypsy elements or interactions between su(Hw) proteins bound to two different su(Hw)-binding regions suppress the insulation and permit the enhancers located between two gypsy elements to activate yellow transcription. The possibility of ectopic intrachromosomal pairing between gypsy elements is supported by the high level of recombination between gypsy sequences: yh alleles arise as a result of recombination between gypsy LTRs. yh derivatives from yh may also be generated by recombination between gypsy sequences as well as between hobo elements.

The prevailing model concerning the mechanism of insulator function proposes that insulators are chromatin boundaries (Geyer and Corces 1992; Harrison et al. 1993; Roseman et al. 1993; Schedl and Grosveld 1995; Gerasimova and Corces 1996). A domain assembled by boundaries prevents interactions between regulatory elements by promoting the folding of a higher-order chromatin structure in such a way as to increase the likelihood of interactions between regulatory elements within a domain, while decreasing these interactions between domains (Vazquez and Schedl 1994). A recent direct finding that blocked enhancers retain their full activity suggests that the effects of the su(Hw) protein on the enhancer function may be caused by the formation of a such domain boundary (Cai and Levine 1995; Scott and Geyer 1995). In view of this, two su(Hw)-binding regions may act as boundaries to define distinct chromosomal domains causing the suppression of insulation seen in yh alleles. Distal enhancers under certain conditions may “bypass” the domain flanked from both sides by su(Hw)-binding regions and activate the proximal yellow promoter. However, this model fails to explain the activation of yellow promoter by enhancers flanked from both sides by a su(Hw)-binding region in the ynh and yh alleles.

Another type of model suggests that the su(Hw)-binding region functions as a flexible regulatory element modulating enhancer-promoter interactions within complex genetic loci (Cai and Levine 1995; Georgiev and Kozycina 1996). Geyer (1997) proposed that insulators assemble complexes that might trap an enhancer in a nonproductive interaction, because the insulator lacks promoter function and no transcription occurs as a result (Decoy model). Other authors postulate that an insulator binding protein interacts and interferes with higher eucaryotic proteins that facilitate interactions between the enhancer and promoter (Morcillo et al. 1996, 1997). The results obtained in the present work may be explained by either model. The ectopic intrachromosomal pairing between two gypsy elements or the interactions between su(Hw) proteins bound to two different su(Hw)-binding regions may prevent the organization of a nonproductive complex between su(Hw) protein and proteins, whose functions are either to activate transcription by enhancer binding or to facilitate the interaction between enhancer and promoter.

On the mechanism of mod(mdg4) gene action: The mod(mdg4) gene encodes a protein that interacts with the su(Hw) protein and contributes to the insulating function of the su(Hw)-binding region (Georgiev and Corces 1995; Gerasimova et al. 1995; Georgiev and Kozycina 1996). In the case of the yh mutation, the hypomorph mod(mdg4)y11 mutation changes the action of the su(Hw)-binding region in such a way that it inactivates yellow transcription driven by enhancers not separated by the su(Hw)-binding region from the yellow promoter. This observation may be explained by assuming that in the presence of the hypomorphic mod(mdg4)y11 mutation, the su(Hw) protein directly inhibits the expression from the yellow promoter (Georgiev and Kozycina 1996). An alternative explanation is that together the su(Hw) and mod(mdg4) proteins are able to affect chromatin structure (Gerasimova et al. 1995; Gerasimova and Corces 1996). According to this hypothesis, binding of the su(Hw) protein to its target sequence creates a bidirectional repressive effect, similar to the silencing caused by heterochromatin. Subsequent interactions between the mod(mdg4) and su(Hw) proteins transform this nonspecific silencer into a polar insulator.

The role of the chromatin structure in the action of mod(mdg4)y11 is supported by the observation that yh, mod(mdg4)y11 males have variegated yellow expression in the tip of the abdomen: dots of a darkly pigmented cuticle against the background of mutant-colored cuticle characteristic of yh flies (Gerasimova et al. 1995). However, we have found here that dots of a darkly pigmented cuticle were absent in males carrying a combination of mod(mdg4)y11 with y alleles that had a deletion of enhancer elements. Therefore, variegated pigmentation on the tip of the abdomen may be interpreted as a result of the ability of enhancer elements to partially overcome su(Hw)-binding insulation in mod(mdg4)y11 background.
In this work, we found that the duplication of gypsy in y"\textsuperscript{a} and y"\textsuperscript{b} alleles completely or partially suppressed the inhibitory effect of the mod(mdg4)\textsuperscript{1u1} mutation on yellow expression in the body and wings. Ectopic intrachromosomal pairing between gypsy elements could alter the properties of the su(Hw)-binding region as an insulator and suppress the effect of the mod(mdg4)\textsuperscript{1u1} mutation. However, it is difficult to explain this fact by assuming that the su(Hw) protein creates a bidirectional repressive effect in the absence of the mod(mdg4) protein. As was shown before, multimerization of sequences only enhanced the possibility of formation of a higher order chromatin structure (Dorer and Henikoff 1994).

The absence of the mod(mdg4)\textsuperscript{1u1} effect on yellow transcription in the yellow-containing construction, where the su(Hw)-binding region is inserted at position --1648 (Georgiev and Kozycina 1996), does not support the possibility that the mod(mdg4)\textsuperscript{1u1} mutation changes the chromatin structure. Although the su(Hw)-binding region in this construction is located between two enhancers of the yellow gene and blocks the wing enhancer (Geyer and Corces 1992), it does not repress yellow transcription in the presence of the mod(mdg4)\textsuperscript{1u1} mutation. This result can hardly be explained in terms of changes of the chromatin structure in the yellow gene by the su(Hw) protein.

The role of the mod(mdg4)\textsuperscript{1u1} mutation with regard to the gypsy insulator was previously studied in transgenic embryos (Cai and Levine 1997). The su(Hw)-binding region was inserted between defined enhancers and placed among divergently transcribed reporter genes (white and lacZ) containing distinct core promoter sequences. The mod(mdg4)\textsuperscript{1u1} mutation caused the insulator to function as a promoter-specific silencer that selectively represses white, but not lacZ. The repression of white does not affect the expression of the closely linked lacZ gene, suggesting that the insulator does not propagate changes in chromatin structure (Cai and Levine 1997).

Thus, the results presented in this work and some previous data support the possibility that the inhibiting action of the mod(mdg4)\textsuperscript{1u1} mutation is realized through a direct interaction of the su(Hw) protein with the yellow promoter, rather than through the action on chromatin structure.

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**LITERATURE CITED**


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