Insertions of Hybrid *P* Elements in the *yellow* Gene of Drosophila Cause a Large Variety of Mutant Phenotypes

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ABSTRACT

A series of *yellow* mutations associated with a great variety of tissue-specific phenotypes were obtained from several highly unstable *Drosophila melanogaster* strains carrying the *gypsy*-induced y^2 allele. These mutations are caused by insertion of additional DNA sequences of variable size 69 bp upstream of the *yellow* transcription start site. These sequences are flanked by identical copies of a deleted 1.2-kb *P* element arranged in the same or inverted orientation. The central part of the inserted element consists of genomic sequences originating from different regions of the *X* chromosome. The mutant phenotype caused by these chimeric elements depends on the nature of the sequences present either in the *P* element or in the central part of the insertion, suggesting that these sequences are able to affect expression of the *yellow* gene. In addition, sequences present in the central region of the insertions strongly modify the effects of the *gypsy*-bound suppressor of Hairy-wing [su(Hw)] and modifier of mdg4 [mod(mdg4)] proteins on *yellow* transcription. Analyses of these mutations give new insights into the mechanisms by which su(Hw) and mod(mdg4) affect enhancer function.

TRANSPOSITION of mobile elements plays an important role in the generation of genotypic and phenotypic diversity in eukaryotes. In *Drosophila melanogaster,* a special role is played by the *P* transposable element family, which is responsible for a syndrome of hybrid dysgenesis that includes chromosome rearrangements, male recombination, high mutability, temperature-sensitive gonadal dysgenesis, and sterility (KIDWELL *et al.* 1977; BINGHAM *et al.* 1982). These genetic abnormalities are due to the mobilization of *P* elements in crosses between males of *P* strains, which carry active *P* elements, and females of M strains devoid of functional *P* elements (ENGELS 1979, 1989; RUBIN *et al.* 1982). The structure of full-length *P* elements is clearly defined, although mobilization of this element results in the accumulation of copies containing deletions of various sizes. In addition, capturing of genomic sequences by *P* elements has also been observed in studies of the *rudimentary* and *vestigial* genes (TSUBOTA and DANG-VU 1991; HESLIP *et al.* 1992).

We have previously described a series of highly unstable mutations in the *ocelliless, white, yellow,* and some other loci that appeared in particular strains after induction of *P*-M hybrid dysgenesis. It was suggested that these mutations also depend on the capture of host sequences by the *P* element (GEORGIEV and YELAGIN 1992). A large number of different mutations with a full spectrum of phenotypes were obtained for each gene. Those affecting the *yellow* (*y*) locus were selected for further studies, as this gene is easily amenable to genetic and molecular analysis. The *yellow* gene is required for pigmentation of larval and adult cuticle and its derivative structures (NASH and YARKIN 1974). The pattern of temporal and spatial expression of the *yellow* gene is controlled by at least five independent tissue-specific enhancer elements (GEYER and CORCES 1987). The body and wing enhancers are located in the 5′ upstream region of *yellow,* whereas tarsal claw and bristle enhancers reside in the intron of the gene.

All unstable *y* mutants appeared in the background of the y^2 allele caused by insertion of the *gypsy* element at −700 bp from the transcription start site of the *yellow* gene (GEYER *et al.* 1986; PARKHURST and CORCES 1986). The y^2 allele displays a tissue-specific mutant phenotype characterized by loss of pigmentation of the wings and body cuticle, whereas all other tissues of the larva and adult show wild-type coloration (NASH and YARKIN 1974). The region of *gypsy* responsible for its mutagenic effect is the binding site for the suppressor of Hairy-wing [su(Hw)] protein (SPANA *et al.* 1988; MAZO *et al.* 1989; DORSETT 1990; SPANA and CORCES 1990). This region 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). A second gene that affects *gypsy*-

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induced phenotypes, *modifier of mdg4* [*mod(mdg4)*], encodes a protein that interacts with su(Hw). Mutations in *mod(mdg4)* enhance the phenotype of y^2 by inactivating *yellow* transcription, probably due to changes in chromatin structure that interfere with the function of enhancers of the *yellow* gene (GEORGIEV and GERASIMOVA 1989; GEORGIEV and CORCES 1995; GERASIMOVA *et al.* 1995).

Here we have molecularly characterized a series of highly unstable *y* mutations and found that they are induced by chimeric elements located 69 bp upstream of the *yellow* transcription start site. The insertions possess a novel and peculiar structure: they are flanked by two identical copies of deleted *P* elements and contain genomic DNA of variable size that was originally located in different regions of the *X* chromosome. Changes in the phenotypes associated with these mutations are accompanied by alterations in either the central part of the insertion or in the *P* elements themselves. The insertion sequences affect transcription from the *yellow* promoter in the mutant gene. In addition, they modulate the effect of mutations in the *su(Hw)* and *mod(mdg4)* genes on *yellow* expression mediated by the *gypsy* retrotransposon also present in the *yellow* locus of these mutants.

MATERIALS AND METHODS

Genetic analyses: All strains were maintained on standard medium at 25°. The alleles and strains used in these studies were described previously (PARKHURST *et al.* 1988; GEORGIEV and GERASIMOVA 1989; GEORGIEV *et al.* 1990; GEORGIEV and YELAGIN 1992; LINDSLEY and GRELL 1992). Highly unstable mutations were obtained and stabilized as described (GEORGIEV and YELAGIN 1992). To induce mutagenesis, three to six males of strains carrying a certain *y* allele and the ry^{506} marker were individually crossed with eight to 10 *C(1)RM, y f;* $P[ry^+\Delta 2\text{-}3](99B)$ females each. Three to 10 F_1 males from every bottle were then individually crossed to eight to 10 *C(1)RM, y f;* ry^{506} females. At least 500 F_2 flies were scored for appearance of derivative *y* alleles. All males with a new phenotype were mated to virgin *C(1)RM, y f;* ry^{506} females. The phenotypes of these *y* alleles were analyzed on 3–5-day-old males. To study the influence of $su(Hw)^2$ and $mod(mdg4)^{u1}$ mutations on the expression of *y* alleles the following crosses were carried out. Males with a *y* allele to be tested were crossed to *C(1)RM, y f* females carrying the *Drop* (*D*) mutation. The F_1 *y; D/+* males were crossed to *C(1)RM, y f;* $su(Hw)^2/T(2;3)ap^{Xa}$ or *C(1)RM, y f;* $mod(mdg4)^{u1}$ females. The F_2 *y; D/*$su(Hw)^2$ or *y; D/*$mod(mdg4)^{u1}$ males were then crossed to *C(1)RM, y f;* $su(Hw)^2/T(2;3)ap^{Xa}$ or *C(1)RM, y f;* $mod(mdg4)^{u1}$ females. Analysis of the phenotype of the *y;* $su(Hw)^2$ or *y;* $mod(mdg4)^{u1}$ 3–5-day-old males was performed at 25°. The results were compared with those obtained in control flies. The pigmentation of four regions of the adult cuticle and its derivative structures, *i.e.*, that of the body, wings, thoracic bristles and abdominal bristles, was analyzed for the phenotypic description of *y* alleles carried out in this paper. In some cases, bristles and hairs of the wing and leg were independently scored. The degree to which the *y* alleles depart from the wild type was determined visually. Wild-type expression was indicated by a score of 5 whereas the absence of *yellow* expression was indicated by a score of 0. Flies with previously characterized *yellow* alleles were used as standards to determine levels of pigmentation and new alleles were examined side by side with those used as standards. For example, a value of 0 corresponds to the pigmentation of the y^- ac^- strain that carries a deletion of the *yellow* gene. A value of 1 for body and wing pigmentation corresponds to that of the y^2 allele (NASH and YARKIN 1974), and a value of 3 corresponds to that of the partial revertant y^{2PR1} (GEORGIEV and KOZICINA 1996). Levels of pigmentation intermediate between y^2 and y^{2PR1} were assigned a value of 2, and levels intermediate between y^{2PR1} and Canton S were assigned a value of 4.

***In situ* hybridization to polytene chromosomes and DNA manipulations:** Drosophila polytene chromosome spreads were prepared from salivary glands of third instar larvae grown at 17°. The preparation of spreads, fixation, denaturation and hybridization were done as described by FAUVARQUE and DURA (1993). Labeling was performed with the random priming kit from Boehringer, using 1 ml of BiodUTP as labeled nucleotide (1 mM biotin-16-dUTP, Boehringer).

Total RNA was isolated from pupal stages of syncronously developing Drosophila cultures by homogenization in 4 M guanidine isothiocyanate, 0.2% *N*-lauroyl sarcosine, 150 mM mercaptoethanol, 12.5 mM EDTA and 50 mM Tris-hydrochloride pH 7.5, followed by phenol extraction and ethanol precipitation (PARKHURST *et al.* 1988). Poly A+ RNA was selected by chromatography on oligo-dT cellulose. Northern analysis was carried out as previously described (PARKHURST *et al.* 1988). Genomic DNA was isolated from adult flies using the protocol described in ASHBURNER (1989). Restriction digests, gel electrophoresis, blotting, cloning and radiolabeling were carried out by standard methods (SAMBROOK *et al.* 1989). DNA sequence analysis was performed by dideoxy chain-termination methodology (SANGER *et al.* 1977). Genomic DNAs were subjected to PCR to amplify sequences from derivative alleles (SAIKI *et al.* 1985; MULLIS and FALOONA 1987). Four different primers from the *yellow* locus (5′ ACTTCCACTTACCATCACGCCAC 3′, 5′ ATGCATTCTATGCACGAGCCTCC 3′, 5′ TCTGTGGACCGTGGCGCGGTAAC 3′, and 5′ CAGCGAAAGGTGATGTCTGACTC 3′) and from the insertion (5′ CGCTGAGGAACTCGAGAAAGGCC 3′, 5′ ACTGCGTGCTTCAAGCTTCTACC 3′, 5′ ATGGCTGCACATAGTCACTGAGC 3′, and 5′ CGTTCCTATTTCCACTACGCAAC 3′) were used to amplify the altered DNA fragment. Amplified DNA was digested and cloned by standard techniques (SAMBROOK *et al.* 1989).

RESULTS

Preliminary characterization of highly unstable yellow alleles: A series of *yellow* mutations were obtained in a strain with a hypermutable *ocelliless* allele in the background of the *gypsy*-induced y^2 mutation. The derivative alleles obtained from the original unstable strain may or may not retain high instability, and some possess the ability to revert with a high frequency to the original mutation (GEORGIEV and YELAGIN 1992; GEORGIEV *et al.* 1992). The phenotypes of the new mutations vary over a wide range, from null in the y^{1s} allele, to a darker than wild-type cuticle coloration observed in the dominant y^{Ds} allele. Phenotypes were cataloged based on the level of pigmentation in four different cuticular structures: body, wings, thoracic bristles and abdominal bristles. As many as 32 different phenotypes were observed due to combinatorial effects in just these four struc-

tures, and some examples of the phenotype of these mutants are shown in Table 1.

Twenty-five highly unstable *yellow* alleles showing a wide range of phenotypes were examined by Southern analysis using various fragments of the *yellow* gene as hybridization probes. Only the *Hin*dIII-*Bam*HI fragment was found to be altered in all highly unstable *y* alleles, suggesting that the mutations are caused by the insertion of foreign sequences within this fragment (Figure 1). All other parts of the *yellow* locus in the mutant strains are indistinguishable from the original y^2 allele. The size of the insertion is different in the various *y* alleles and varies from 1.2 to >20 kb, as deduced from Southern analyses in which genomic DNAs were digested with *Bam*HI or *Kpn*I endonucleases (data not shown). On the other hand, the ends of the insertions responsible for these highly unstable mutations were rather uniform. All of them have restriction sites for *Hin*dIII and *Xho*I, and they can be classified into two types that differ in the restriction pattern for these two enzymes (Figure 1). One *y* allele from each group was selected for further molecular analysis: y^{+ns}, containing an insertion ~21 kb in length, and y^{+s}, which carries an insertion of ~5.3 kb.

Structure of the highly unstable y^{+ns} mutation: The y^{+ns} allele displays normal pigmentation of the body and wings but yellow bristles on the notum and legs (see Table 1). To understand the molecular basis of this phenotype, we cloned fragments of y^{+ns} genomic DNA hybridizing to the *Hin*dIII-*Bam*HI fragment of the wild-type *yellow* locus. Five overlapping phage clones from both sides of the insertion were obtained. Restriction maps of the clones coincide with those deduced from Southern blot analysis of genomic DNA, confirming the absence of artificial recombinant clones. DNA sequence analysis of the insertion boundaries indicates that the insertion is located 69 bp upstream to the *yellow* transcription start site (Figure 1); in addition, *yellow* sequences from −146 to −70 bp are deleted. The insertion contains a central region of 19.7 kb flanked by two identical copies of a 1.2-kb deleted *P* element arranged in the same orientation; the total size of the insertion is 22.1 kb. The direction of transcription of the *P* element is opposite to that of the *yellow* gene. The flanking *P* elements contain a deletion from 830 to 2430 bp and a new decanucleotide sequence TAGCTACAAA at the breakpoint of this deletion. This sequence could not be found among published *P*-element sequences. The two *P* elements were designated as P1 and P2 depending on whether they are located distal or proximal to the *yellow* promoter, respectively. Neither the whole insertion nor the individual *P* elements are framed by the characteristic base pair duplications that normally appear after *P*-element insertion, suggesting that further rearrangements took place after the initial insertion of *P*-element sequences.

To gain insights into the nature of the core sequences flanked by *P* elements in the insertion responsible for the y^{+ns} phenotype, we carried out Southern analyses using genomic DNA from these flies and various restric-

TABLE 1

Pigmentation of y^2 derivatives and interactions with *su(Hw)* and *mod(mdg4)* mutations

yellow allele	Pigmentation																	
							Bristles											
	Body			Wings			Th			Leg			Wing			Abdomen		
	wt	*su(Hw)*	*mod*	wt	*su(Hw)*	*mod*	wt	*su(Hw)*	*mod*	wt	*su(Hw)*	*mod*	wt	*su(Hw)*	*mod*	wt	*su(Hw)*	*mod*
y^2	1	5	0	1	5	0	5	5	0	5	4	0	5	5	0	5	5	0
y^{+ns}	5	3	4	5	3	4	0	0	0	2	2	0	5	5	3	5	5	3
y^{+lnsx1}	3	3	3	3	3	3	0	0	0	1	1	1	5	5	5	5	5	5
$y^{+mws1,2,3}$	1	0	0	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0
y^{+lws1}	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
$y^{2s1,2,3}$	1	5	0	1	5	0	5	5	0	5	4	0	5	5	0	5	5	0
$y^{2s4,5}$	1	0	0	1	0	0	5	0	0	5	0	0	5	2	2	5	3	3
y^{+s}	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
$y^{+s11,12}$	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
$y^{+s22,23}$	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
$y^{+ls1,2}$	3	5	3	3	5	3	5	5	5	5	5	5	5	5	5	5	5	5
$y^{2s8,21,10-2}$	1	5	0	1	5	0	5	5	0	5	4	0	5	5	0	5	5	0
y^{ds66}	2	5	2	2	5	2	5	5	5	5	5	5	5	5	5	5	5	5

Numbers indicate levels of pigmentation, with 5 corresponding to wild-type levels and 0 indicating the pigmentation of a null *yellow* allele. Bristles are subdivided into thoracic (Th), leg (L), wing (W) and abdominal (Ab).

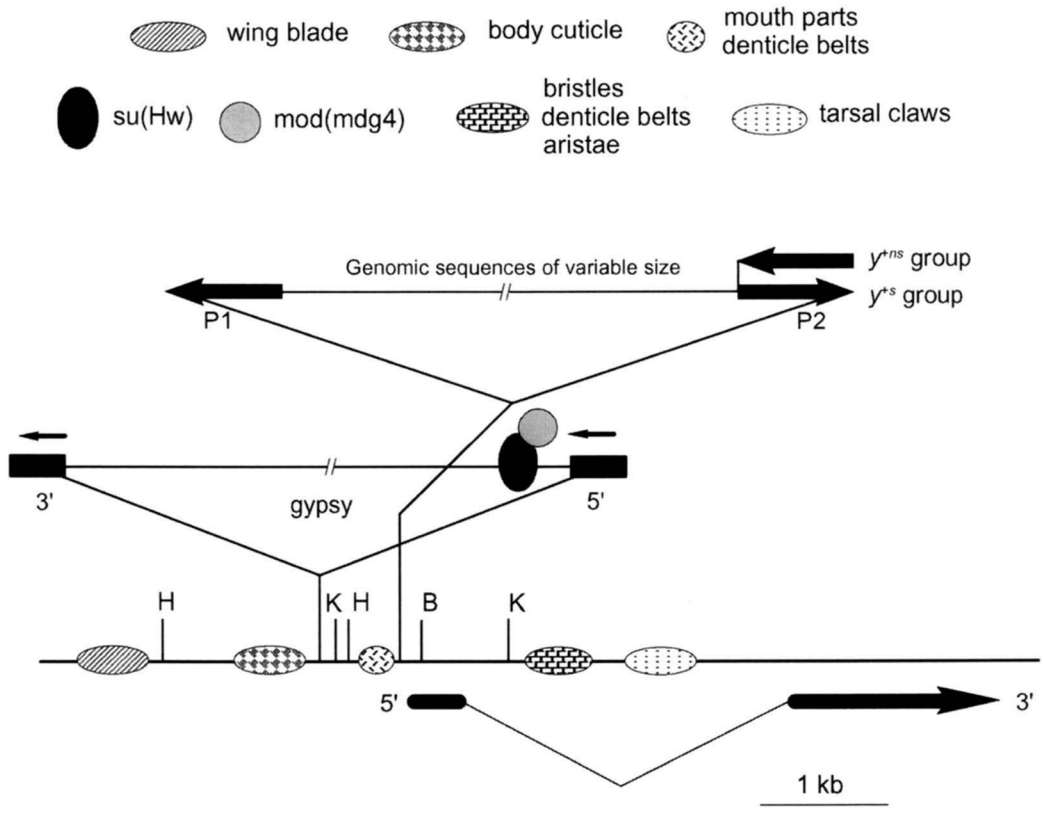

FIGURE 1.—Schematic representation of the structure of the *yellow* locus and highly unstable *yellow* alleles. The structure of the *yellow* RNA is indicated by thick lines denoting the exons and a thin line denoting the intron; the direction of transcription is indicated by arrows. The *gypsy* element is inserted at −700 bp from the *yellow* transcription start site both in y^2 and its hyperunstable derivatives; arrows indicate the extent and orientation of the long terminal repeats (LTRs), a solid oval denotes the su(Hw) protein and a stippled circle indicates the mod(mdg4) protein. Ovals throughout the *yellow* gene indicate tissue-specific transcriptional enhancers responsible for *yellow* expression in various tissues; the tissues where each of these enhancers are active are indicated in the top part of the figure. Arrows in the insertion sequences indicate the size and orientation of *P*-element sequences present in hypermutable *yellow* alleles. Restriction sites for *Hin*dIII (H), *Kpn*I (K) and *Bam*HI (B) present in the *yellow* gene are also indicated.

tion fragments as hybridization probes (Figure 2). The *Pst*I fragment b in Figure 2A hybridizes to multiple bands on Southern blots, suggesting that sequences located adjacent to the P1 *P* element are repeated in the genome (Figure 2B). The extent of these repetitive sequences must be quite limited, since the intensity of the bands in the Southern blot corresponding to the repeated DNA is much lower than those hybridizing to single copy sequences; a similar but not identical pattern of DNA bands was obtained with different *D. melanogaster* strains (Figure 2B). Multiple bands indicating the presence of repetitive sequences are also observed in Southern blots using the *Eco*RI-*Sac*I fragment c or *Sac*I-*Eco*RI fragment d as hybridization probes (Figure 2, C and D). All other fragments of the insertion hybridize just with single copy bands of genomic DNAs from the different strains analyzed. For example, the *Hin*dIII-*Eco*RI fragment e hybridizes to one band on genomic blots (Figure 2E), and the same is true for *Sac*I fragments f and g (data not shown). These results suggest that most of the central region of the insertion responsible for the y^{+ns} phenotype is present in very low copy number in the genome, and it is therefore unlikely to correspond to a transposable element. To determine the origin of these sequences, we carried out *in situ* hybridization to polytene chromosomes from third instar larvae. Probe f hybridizes to the 1A and 11F chromosomal subdivisions on Oregon R polytene chromosomes, whereas probe g hybridizes to 11A and 11F (Figure 2, F and G). Thus, the y^{+ns} insertion contains sequences from two, or possibly three, different regions of the *X* chromosome.

Molecular analysis of the y^{+s} allele: A second highly unstable y^2 derivative, y^{+s}, shows a wild-type phenotype (see Table 1). Southern blot hybridization confirms the presence of an insertion ~5.3 kb long in the same position as in y^{+ns}. Cloning and sequencing of the insertion have shown that it contains a central region flanked by copies of a 1.2-kb *P* element arranged in opposite orientations (Figure 3A). The *P* element closer to *gypsy*, P1, has exactly the same sequence and orientation as the P1 element in the y^{+ns} allele. In contrast to y^{+ns}, the P2 element in y^{+s} is arranged opposite to P1. Again, neither the whole insertion nor individual *P* elements

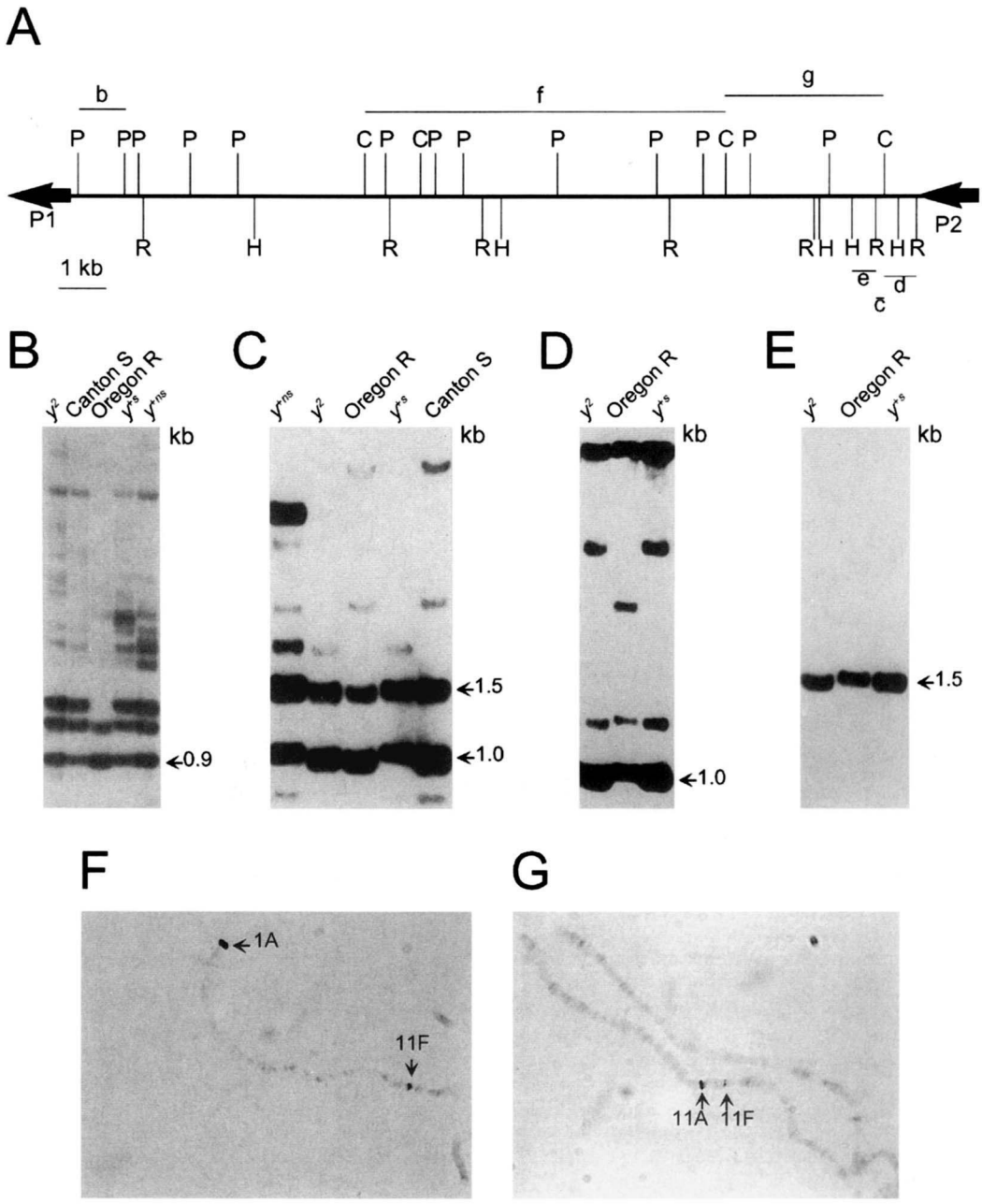

FIGURE 2.—Structure and genomic distribution of the y^{+ns} insertion. (A) Restriction map of the y^{+ns} insertion; only part of the *P* elements are shown. Restriction enzyme symbols are R, *Eco*RI; H, *Hin*dIII; C, *Sac*I; P, *Pst*I. The DNA fragments used for Southern blot and *in situ* hybridization are indicated by horizontal lines. (B) Southern analysis of repetitive sequences adjacent to the P1 element. DNA from y^2, Canton S, Oregon R, y^{+s} and y^{+ns} was digested with *Sac*I and *Pst*I, and the blot was probed with *Pst*I fragment b; the band corresponding to the internal region of the insertion is indicated by an arrow. (C) Southern analysis of repetitive sequences adjacent to the P2 element. DNA from y^2, Oregon R, y^{+s}, y^{+ns} and Canton S was digested with *Sac*I and *Pst*I, and the blot was probed with *Eco*RI-*Sac*I fragment c. (D) Southern analysis of single copy sequences located in the y^{+ns} insertion. DNA from y^2, Oregon R and y^{+s} was digested with *Sac*I and *Pst*I, and the blot was probed with *Sac*I-*Eco*RI fragment d. (E) Same blot as Figure 2D after hybridization with *Hin*dIII-*Eco*RI fragment e. (F) Localization of internal regions of the y^{+ns} insertion on polytene chromosomes of Oregon R probed with *Sac*I fragment f. (G) Localization of internal regions of the y^{+ns} insertion on polytene chromosomes of Oregon R probed with *Sac*I fragment g. Arrows indicate the location of hybridization.

are framed by duplications of genomic sequences. Four terminal nucleotides are missing from the 5′ end of P2.

The central part of the y^{+s} insertion is composed of 2.9 kb of genomic sequences as deduced from Southern blot hybridization and direct DNA sequencing. The *Hin*dIII-*Eco*RI DNA restriction fragment adjacent to P1 contains repetitive sequences present several times in the genome and located at the same position in several different Drosophila strains (Figure 3B). All other parts of the insertion consist of unique genomic sequences (Figure 3C) captured from the 1A region of the *X* chromosome. The original location of these sequences is

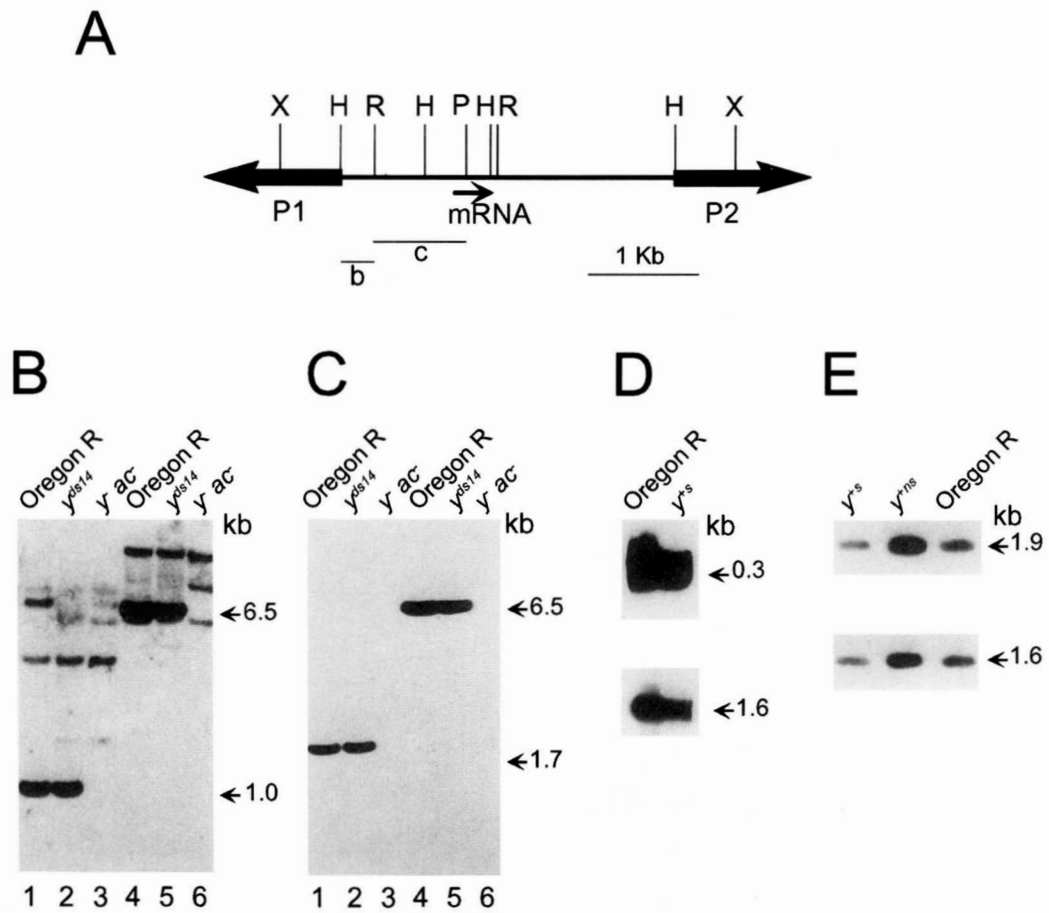

FIGURE 3.—Structure and organization of the y^{+s} insertion. (A) Restriction map of the y^{+s} insertion. *P* elements are indicated by solid arrows. Restriction enzyme symbols are R, *Eco*RI; H, *Hin*dIII; X, *Xho*I; P, *Pst*I. DNA fragments used for Southern analysis are indicated by horizontal lines. The localization of the transcript encoded by sequences present in the insertion is marked by an arrow. (B) Southern analysis of genomic DNA from the Oregon R, y^{ds14} and y^{-} ac^{-} strains digested with *Hin*dIII/*Eco*RI (1–3) or *Bgl*II (4–6) and probed with *Hin*dIII-*Eco*RI fragment b. (C) Same blot after striping and subsequent hybridization with the *Eco*RI-*Pst*I fragment c. (D) Analysis of the insertion-encoded transcript on Northern blots of RNA isolated from y^{+s} and Oregon pupae. The blot was hybridized with a 150-bp PCR fragment spanning the transcribed region (top). The 300-bp RNA is flanked by putative promoter sequences located 1144 bp from the end of the P1 element, and a polyadenylation site located 1501 bp from the same end. As a control, the blot was also hybridized with a *ras2* probe that detects a 1.6-kb transcript (bottom). (E) Northern analysis of *yellow* expression during midpupal stages of development. Poly A+ RNAs were isolated from y^{+s}, y^{+ns} and Oregon R strains; ^{32}P-labeled DNA fragments containing the *yellow* and *ras2* genes were used as hybridization probes. The *yellow* probe hybridizes to a 1.9-kb RNA (top), whereas *ras2* gives rise to a 1.6-kb transcript that is expressed at approximately constant levels during Drosophila development and is shown as a marker for the amount of RNA (bottom).

in close proximity and distal to the *yellow* locus. This conclusion is based on the observation that genomic DNA from the y^{-} ac^{-} strain, which contains a deletion extending 5 kb from the *yellow* gene in the distal direction (GEYER *et al.* 1990), does not hybridize with probes from the insertion, whereas DNA from flies carrying a deletion extending only 3 kb upstream of the *yellow* gene, y^{ds14}, does (Figure 3, B and C). DNAs from the insertion sequences responsible for the y^{+ns} and y^{+s} mutations do not cross hybridize, suggesting that the insertions present in these mutants, with the exception of the *P* element, contain completely different genomic sequences. DNA sequence analysis of the y^{+s} insertion suggests that the duplicated genomic region flanked by *P* elements contains a small putative gene, including a short open reading frame flanked by a TATA box and a polyadenylation site (Figure 3A). Northern analysis using the central part of the insertion sequence as a probe indicates the presence of a relatively abundant RNA hybridizing to this sequence that is expressed during pupal stages of development, at the time when the *yellow* gene is also transcribed (Figure 3D).

Expression of the yellow gene in the y^{+ns} allele and its derivatives: The y^{+ns} mutation was obtained in the background of the *gypsy*-induced y^{2} allele. In y^{2}, *yellow* transcription in the body and wings is blocked by the su(Hw)-binding region of *gypsy* that separates *yellow* enhancers, controlling the expression of the gene in the two mutant tissues, from the promoter. The strong hypomorph $su(Hw)^{2}$ mutation suppresses the mutant y^{2} phenotype (HARRISON *et al.* 1993), whereas the $mod(mdg4)^{u1}$ mutation has the opposite effect, causing partial

or complete inactivation of *yellow* expression in all cuticular structures of the fly (GEORGIEV and GERASIMOVA 1989). The y^{+ns} allele has an insertion of additional sequences between the *gypsy* element and the promoter, but flies carrying this mutation show a reversion of the wing and body cuticle phenotype of y^2. This reversion of the mutant phenotype could be due to activation of transcription from a promoter located in the y^{+ns} insertion, or to an interference of this insertion with the ability of the su(Hw)-binding region present in *gypsy* to insulate enhancer-promoter interactions.

To understand the molecular basis of the mutant phenotype caused by the insertion present in the y^{+ns} allele, we analyzed the effect of the *su(Hw)* and *mod(mdg4)* mutations on the phenotype of the y^{+ns} allele. Mutations in *mod(mdg4)* partially decrease the pigmentation of all cuticular structures but, unexpectedly, the $su(Hw)^2$ mutation reduces the body and wing pigmentation of y^{+ns} flies without affecting bristle pigmentation (Table 1). Thus, the effect of the su(Hw) protein seems to be opposite to that observed in y^2, possibly as a result of the presence of new regulatory sequences within the y^{+ns} insertion. One explanation for this result could be the presence of new su(Hw)-binding sites in the y^{+ns} insertion. To test such possibility, we used X-ray irradiation of y^{+ns} flies to obtain a derivative, named y^{+1nsX1}, in which *gypsy* has been excised by recombination between the LTRs. Flies carrying this mutation have a less severe body and wing phenotype than y^2, and this phenotype is identical to that of y^{+ns} in combination with $su(Hw)^2$ (Table 1). The presence of the $su(Hw)^2$ or $mod(mdg4)^{u1}$ mutations fails to change the level of expression of the *yellow* gene in flies carrying the y^{+1nsx1} allele, suggesting that the remaining insertion does not contain su(Hw)-binding sites and its effect on *yellow* expression in y^{+ns} mutants might be due to interference with the ability of the su(Hw) insulator to affect enhancer-promoter interaction. In addition, these results suggest that, in the presence of an additional 22.1-kb insertion between the *gypsy* element and the promoter, the su(Hw)-binding region has a positive rather than a negative effect on *yellow* transcription.

Additional information on the molecular basis of the y^{+ns} phenotype was obtained by analysis of several y^{+ns} derivatives to determine the nature of putative regulatory regions present within the insertion. These derivatives were induced by crosses between y^{+ns} and flies carrying the P[ry^+ Δ2-3](99B) transposon as an autonomous source of transposase to induce mobilization of *P* elements flanking the insertion (ROBERTSON *et al.* 1988). Derivative alleles of the y^{+ns} offspring were grouped into different categories based on phenotypic characteristics (Table 1). Four of these alleles, named y^{+mws1}, y^{+mws2}, y^{+mws3} and y^{+1ws1}, show similar phenotypes characterized by very low levels of pigmentation of the wings and body cuticle, and complete lack of coloration of the bristles (Table 1). Southern blot analysis indicates that these mutations differ from y^{+ns} only in the P2 region. The altered P2 element from these four alleles was cloned by PCR and sequenced. All resulted from internal deletions in the P2 element (Figure 4). The complete central region of the P2 element is deleted in y^{+mws1}, y^{+mws2}, and y^{+mws3}, with the exception of 13–16 bp of the flanking inverted repeats. The y^{+1ws1} allele has an internal deletion of P2 element sequences with breakpoints at nucleotides 16 and 2695. As in the case of y^{+ns}, both $su(Hw)^2$ and $mod(mdg4)^{u1}$ mutations enhance the phenotype of named y^{+mws1}, y^{+mws2}, y^{+mws3} and y^{+1ws1} alleles in the body and wings. For example, flies with y^{+1ws1} and $su(Hw)^2$ or $mod(mdg4)^{u1}$ have a phenotype close to y^1, *i.e.*, complete inactivation of the *yellow* gene. These results suggest that sequences contained within the P2 element are directly or indirectly responsible for the activation of *yellow* transcription in the y^{+ns} mutation with respect to y^2. Northern analysis of pupal RNA shows that y^{+ns} flies accumulate a transcript of the same size as Oregon R (Figure 3E), suggesting that transcription takes place from the normal *yellow* promoter or from an ectopic promoter located nearby; this promoter sequence could be located in the upstream P2 element.

A third class of mutations display normal pigmentation of the bristles and mutant of body and wings, similar to y^2. Five different alleles of this class were obtained, and molecular analysis indicates that these mutations are caused by deletions in the central part of the original insertion (Figure 4). In three of them, y^{2s1}, y^{2s2}, and y^{2s3}, the phenotype is suppressed by $su(Hw)^2$ and enhanced by $mod(mdg4)^{u1}$, just as in the case of the y^2 allele. These $y^{2s1\text{-}3}$ alleles have deletions similar in size (~6.2 kb) and localization (Figure 4). Thus, the residual part of the insertion fails to influence *yellow* expression and to change the effect of the su(Hw)-binding region, suggesting that sequences that interfere with the su(Hw) insulator are located within the deleted region. Two additional alleles, y^{2s4} and y^{2s5}, have different properties: both $su(Hw)^2$ and $mod(mdg4)^{u1}$ mutations decrease the pigmentation of the bristles and fail to affect the pigmentation of the body and wings. Southern blot analysis shows that these alleles contain smaller 5-kb deletions that represent a subset of the 6.2-kb deletion observed in the, y^{2s1}, y^{2s2}, and y^{2s3} alleles. These results suggest that sequences present in the central part of the insertion might interfere with the insulating ability of the su(Hw) protein. In addition, the 1.2-kb fragment of DNA, representing sequences deleted in y^{2s1}, y^{2s2} and y^{2s3} but present in y^{2s4} and y^{2s5}, and located at the left side of the 6.2-kb deletion in Figure 4, is responsible for causing a new kind of interaction between the su(Hw) binding region and *yellow* gene expression.

Expression of the yellow gene in the y^{+s} allele and its derivatives: Flies carrying the y^{+s} allele have normal pigmentation of all cuticular structures, and this pattern of pigmentation is not altered by either *mod(m-*

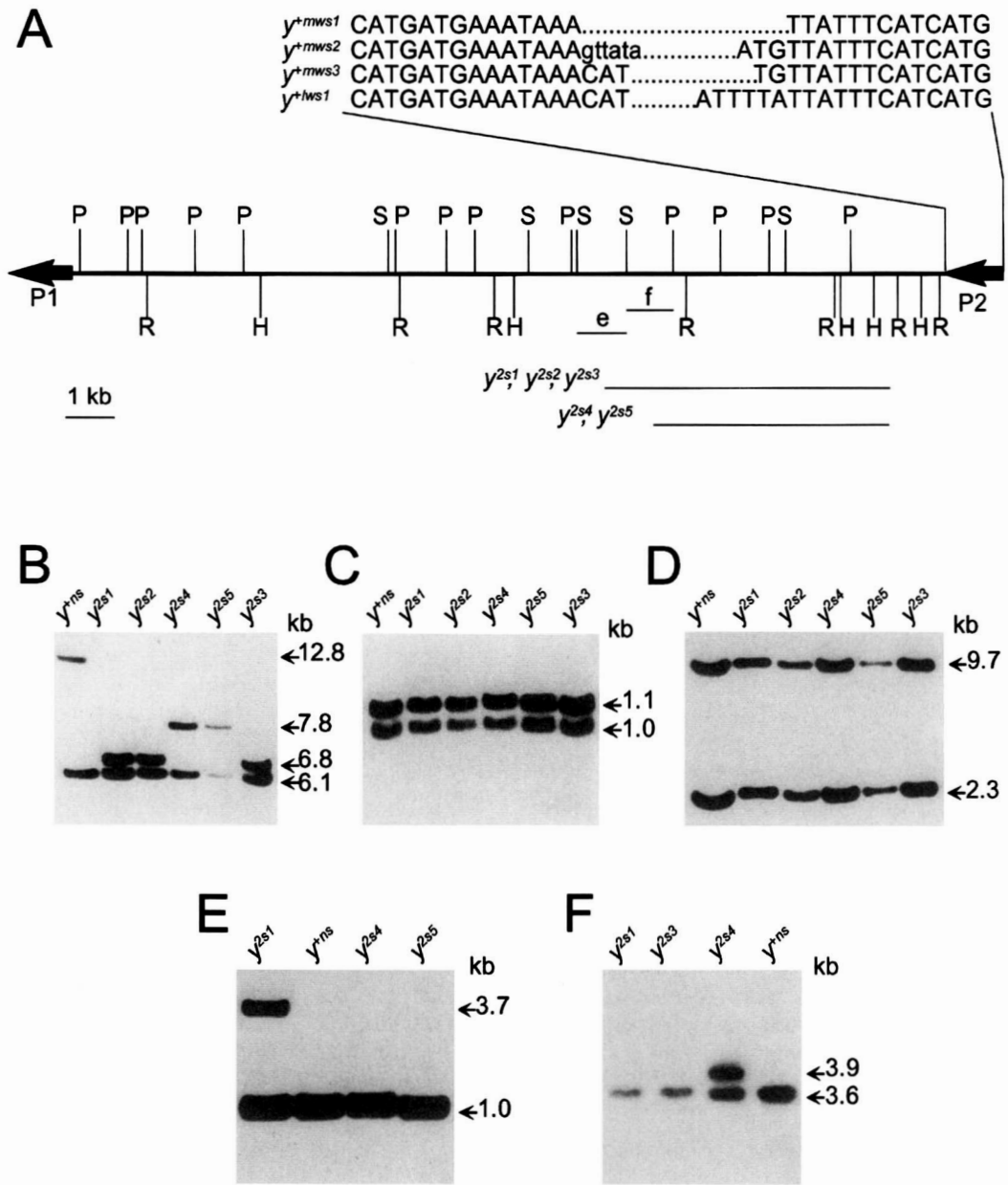

FIGURE 4.—Structure of y^{+ns} derivative alleles. (A) Schematic representation of the y^{+ns} insertion and alterations responsible for various derivatives. Thin lines show the extent of deleted sequences in the y^{2s1}, y^{2s2}, y^{2s3}, y^{2s4} and y^{2s5} derivatives. The precise location of the breakpoints was determined by Southern analysis followed by PCR cloning and sequencing. DNA fragments used for Southern analysis are indicated by horizontal lines. Sequences remaining after excision of the P2 element in three y^{+mws} and one y^{+lws} derivatives are indicated in the upper part of the diagram. (B) Southern analysis of genomic DNA from flies carrying the y^{+ns}, y^{2s1}, y^{2s2}, y^{2s3}, y^{2s4} and y^{2s5} mutations; the DNAs were digested with *Kpn*I and hybridized with the *Hin*dIII-*Bam*HI fragment from the *yellow* locus (see Figure 1). (C) DNAs were digested with *Bam*HI/*Xho*I and hybridized with the *Hin*dIII-*Bam*HI fragment from the *yellow* locus. (D) DNAs were digested with *Bam*HI-*Sac*I and hybridized with the *Hin*dIII-*Bam*HI fragment from the *yellow* locus. (E) Genomic DNAs from y^{2s1}, y^{+ns}, y^{2s4} and y^{2s5} flies were digested with *Sal*I and subjected to Southern analysis using the *Sal*I fragment e as a probe. (F) Genomic DNAs from y^{2s1}, y^{2s3}, y^{2s4}, and y^{+ns} were digested with *Sal*I and hybridized to the *Sal*I-*Pst*I fragment f.

dg4)^{u1} or *su(Hw)*^{2} mutations (Table 1). Northern blot analysis shows that the size and amount of *yellow* transcript at the pupal stages of development, when *yellow* gene expression determines the pigmentation of adult cuticle, are the same in y^{+s} and in control Oregon R strains (Figure 3E). Derivatives of y^{+s} were obtained by crosses with the P[ry^{+} Δ2-3](99B) strain. Two alleles, y^{+s11} and y^{+s12}, are caused by an inversion of the central region without deletion of P2 element sequences. We also obtained two y^{+s} derivatives (y^{+s22} and y^{+s23}) by a specific genetic screen (T. BELENKAYA, unpublished data) (Figure 5). These four alleles are caused by an inversion of the central part of the insertion. No phenotypic differences between the original y^{+s} flies and its

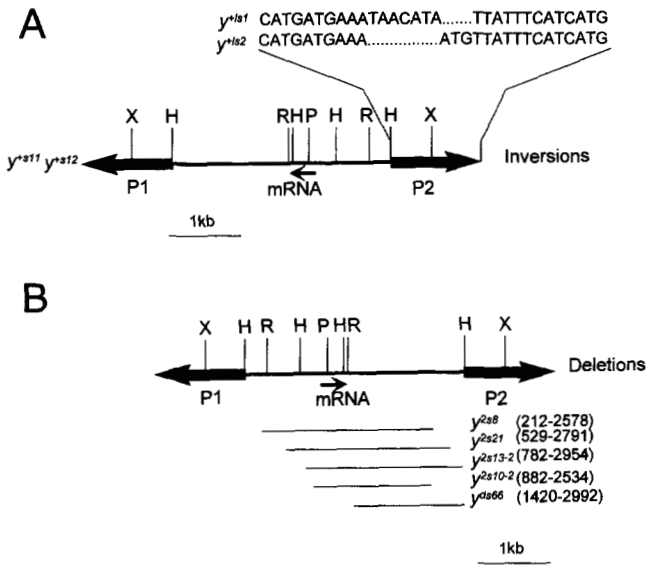

FIGURE 5.—Schematic representation of y^{+s} derivatives. Restriction map of the y^{+s} mutation and derived alleles. (A) The diagram displays the structure of alleles caused by inversion between *P*-element ends. The y^{+s11} and y^{+s12} alleles are caused by a simple inversion; the y^{+ls1} and y^{+ls2} alleles contain additional deletions of P2 element sequences. (B) The diagram displays the structure of mutations induced by deletions of the central region of the insertion. The breakpoints of the deletions responsible for the y^{2s13-2}, y^{2s10-2}, y^{2s21}, y^{2s8} and y^{ds66} alleles were cloned by PCR and sequenced. Thin lines indicate the extent of the deleted sequences, and numbers in parenthesis represent the exact breakpoints within the sequence of the insertion.

derivatives were found, suggesting that the effect of the central region of the insertion on *yellow* expression acts in an orientation-independent fashion. Two additional alleles isolated, named y^{+ls1} and y^{+ls2}, are characterized by a lower pigmentation of the wings and bristles. DNA sequence analyses indicate they were induced by simultaneous inversion of the whole central part of the insertion and internal deletion of almost all P2 element sequences (Figure 5). These results suggest that *P*-element sequences contribute to normal expression of the *yellow* gene in the y^{+s} allele. The contribution of the residual sequences present in the y^{+ls1} and y^{+ls2} mutations to the *yellow* phenotype observed in flies carrying these mutations was determined by examining their phenotype in the background of mutations in *su(Hw)* and *mod(mdg4)*. The $su(Hw)^2$ mutation completely suppresses the phenotype of the y^{+ls1} and y^{+ls2} alleles, whereas $mod(mdg4)^{u1}$ has no effect. This result suggests that sequences remaining in the y^{+ls1} and y^{+ls2} mutations do not have a negative effect on *yellow* expression.

Five additional derivatives of y^{+s} were obtained in the crosses described above; these y^{2s} alleles show a phenotype similar to y^2 and are associated with deletion of internal sequences of the y^{+s} insertion. The ends of the deletions were cloned by PCR and sequenced (Figure 5). Four alleles, y^{2s8}, y^{2s21}, y^{2s10-2} and y^{2s13-2}, displayed the same phenotype as y^2, and responded to mutations in *su(Hw)* or *mod(mdg4)* in the same manner as y^2 (Table 1). All these mutations are caused by internal deletions of the insertion responsible for the y^{+s} mutation; these deletions include the coding region of the putative gene present in this insertion (Figure 5). An additional allele isolated in these experiments, y^{ds66}, shows a phenotype similar to y^2; this phenotype is completely suppressed by $su(Hw)^2$, but not enhanced by the $mod(mdg4)^{u1}$ mutation (Table 1). In contrast to other y^{2s} alleles, this mutant contains a shorter deletion that does not affect sequences of the insertion encoding a transcript expressed during pupal development. This suggests that the central part of the insertion might contain two types of regulatory elements. One of them activates *yellow* transcription in the wing and body cuticle or interferes with the effect of the su(Hw) insulator on *yellow* expression in these tissues. A second one suppresses the bidirectional silencing effect of su(Hw) in the absence of mod(mdg4) protein. The latter roughly coincides with the region containing promoter elements of a gene present in the insertion.

DISCUSSION

The y^2 mutation results from the insertion of the *gypsy* element in the 5′ region of the *yellow* gene. The phenotype of this mutation is caused by the inability of enhancers located upstream of the insertion site to act on the *yellow* promoter due to the presence of the su(Hw) protein bound to *gypsy* sequences. This protein, and the associated mod(mdg4) product, cause this effect on transcription by creating a chromatin insulator and segregating upstream enhancers from the promoter by locating each of these elements into separate higher order domains of chromatin organization. Insertion of additional sequences between the *gypsy* element and the promoter alter this phenotype in a complex manner that depends on the nature of the insertion. Two of these y^2 derivatives have been analyzed in detail and both were found to contain *P*-element sequences flanking a central region captured from a different genomic location. Insertions of hybrid *P* elements have also been found at the *rudimentary* (*r*) and *vestigial* (*v*) genes. The r^{hd4} allele is caused by insertion of a *P* element and adjacent genomic sequences that are able to transpose as a unit (TSUBOTA and DANG-VU 1991), whereas the vg^{28w} mutation was caused by insertion of a *P*[*Ddc*] transposon containing 9.5 kb of additional sequences captured from chromosomal subdivision 55C (HESLIP *et al.* 1992). The insertions responsible for the y^{+ns} and y^{+s} mutations are different from previously described hybrid *P* elements in that they consist of a central core flanked by two identical *P* elements. The arrangement of these sequences is reminiscent of composite bacterial transposons, such as *Tn10*, that contain an antibiotic-resistance gene flanked by two *IS* ele-

ments. In spite of this structural similarity, it is not clear that the hybrid *P* elements found in the *yellow* gene are able to move as a unit, since the complete insertion is not flanked by identical direct repeats. A more plausible explanation is that a *P* element and adjacent sequences are inserted in the 5′ region of the *yellow* gene in an event similar to that described by TSUBOTA and DANG-VU (1991); a second insertion of similar sequences a few nucleotides upstream followed by recombination of intervening sequences could explain both the lack of flanking repeats and the deletion of *yellow* sequences between −146 and −70.

The presence of this composite *P* element in the 5′ region of the *yellow* gene results in highly unstable mutations that give rise to derivatives displaying a variety of mutant phenotypes. These phenotypes are the result of a complex series of effects on *yellow* expression caused by the presence of *P*-element sequences, regulatory sequences within the captured central core, and interactions between these sequences and the su(Hw) insulator present in the adjacent *gypsy* element. The contribution of the composite *P* element to the final phenotype can be determined by examining the coloration of flies carrying a mutation in the *su(Hw)* gene. In addition, partial deletion of the inserted sequences has allowed us to determine the contribution of *P*-element sequences *vs.* the captured central core to the observed *yellow* phenotype. Excisions of the P2 element, located proximal to the *yellow* promoter, are associated with a reduction of *yellow* expression, whereas deletions affecting the P1 element have no effect. Deletions affecting the P2 element leave behind 10–17 bp of the *P*-element inverted repeat; these sequences have been shown to inhibit *yellow* expression when localized close to the *yellow* promoter (A. OGANESIAN and P. GEORGIEV, unpublished observations). These sequences interact with the inverted repeat binding protein (IRBP) (RIO and RUBIN 1988), and this interaction could result in decreased transcription from the promoter of the *yellow* gene.

A detailed analysis of the nature of the core sequences flanked by the two *P* elements has given additional insights into the molecular basis for the complex phenotype of the resulting y^2 derivatives. The y^{+ns} mutation is caused by a large 22.1-kb insertion at −69 bp from the transcription start site. The presence of these sequences reverses the mutant effect of *gypsy*-bound su(Hw) on the body and wing enhancers, while the coloration of the bristles and wing margin remain wild type. This suggests that the inserted sequences either interfere with the insulating function of su(Hw), carry a cryptic promoter that can drive *yellow* expression in the appropriate tissues, or positively regulate the normal *yellow* promoter. Northern analysis indicates that *yellow* RNA present in these mutant flies during pupal development is the same size as wild type, suggesting that it is transcribed from the normal *yellow* promoter or, if a promoter is present in the insertion, it must be located in the P2 copy of the *P* element closest to the *yellow* gene. Additional sequences present within the y^{+ns} insertion contribute to the overall pattern of expression of the *yellow* RNA in this mutant. Mutations that delete a 6.2-kb region within the central part of the insertion in the y^{2s1}, y^{2s2} and y^{2s3} alleles result in an increase of the y^{+ns} phenotype to that typical of y^2. This suggests that sequences contained within this 6.2-kb region might also play a regulatory role to activate *yellow* expression in the wings and body cuticle. Alternatively, these sequences might exert this effect by interfering with the insulating properties of su(Hw). Analysis of two additional mutants, y^{2s4} and y^{2s5}, allows further subdivision of these sequences and the identification of a 1.2-kb region that has itself the properties of a su(Hw)-dependent silencer that causes repression in all tissues in the absence of su(Hw). The mechanism by which *P*-element sequences affect *yellow* expression in the y^{-ns} mutation is unclear. The P2 element could contain promoter sequences that allow initiation of transcription continuing into the *yellow* gene. Alternatively, the *P* element could contain enhancer sequences that could activate *yellow* expression in the appropriate tissues at the right time of development. Finally, the insertion sequence responsible for the y^{+ns} mutant phenotype could form an abnormal DNA structure as a consequence of the two tandemly repeated copies of the *P* element flanking the insertion. This structure could interfere with changes in chromatin structure that presumably are the basis of the process by which the su(Hw) insulator represses the function of the wing and body cuticle enhancers.

A similar situation can be observed in the case of the y^{+s} allele. Flies carrying this mutation display a wild-type phenotype, suggesting that the insertion sequences responsible for the mutation carry regulatory elements that activate *yellow* expression in the wings and body cuticle or the insertion interferes with the insulating effect of su(Hw). The latter effect could be caused by the formation of a hairpin structure between the two inverted copies of the *P* elements that flank the insertion. This structure could interfere with the formation of higher order domains of chromatin that are the basis for the insulating effect of su(Hw). This hypothesis is supported by the structure of one type of y^{+s} derivative: deletion of P2 element sequences in y^{+1s1} and y^{+1s2} results in decreased *yellow* expression in the wings and body cuticle and this effect is reversed by mutations in su(Hw). Inversions of the central region of the insertion in the y^{+s11} and y^{+s12} derivatives do not affect the expression of the *yellow* gene, indicating that the orientation of the internal sequences of the insertion is inconsequential to the phenotype. This result suggests that these sequences do not contribute promoter elements that affect transcription of the *yellow* gene, but rather it might be the structure of the hairpin that is responsi-

ble for the reversion of the insulating effect of su(Hw). In support of this idea, deletion of large regions of the loop of this hairpin in the y^{2s8}, y^{2s21}, $y^{2s13\text{-}2}$, $y^{2s10\text{-}2}$, and y^{ds66} derivatives results in repression of *yellow* expression in the wings and body cuticle. This effect is again probably caused by a restoration of the functionality of the su(Hw) insulator as a consequence of the large deletions in the hairpin structure located between the su(Hw) binding region and the promoter. Interestingly, mutations in *mod(mdg4)* do not affect the coloration of the bristles in the original y^{+s} mutation or in most of its derivatives. It has been proposed that the repression of *yellow* expression in all tissues observed in the background of mutations in *mod(mdg4)* is caused by a bidirectional silencing effect caused by the su(Hw) protein in the absence of mod(mdg4) (GERASIMOVA *et al.* 1995). This effect extends at least 15 kb from a *gypsy* element located in the *achaete-scute* complex to the bristle enhancer located in the *yellow* gene, but it does not affect enhancer elements located in the 5′ region of the *yellow* gene and separated by the promoter from the *gypsy* element (GEORGIEV and KOZYCINA 1996). The same situation can be observed in several derivatives of the y^{+s} allele. Mutations such as $y^{2s13\text{-}2}$, $y^{2s10\text{-}2}$, y^{2s21}, and y^{2s8}, in which the promoter and coding sequences of a putative gene present in the central region of the insertion have been deleted, respond to mutations in *mod(mdg4)* by repressing *yellow* expression in the bristles. To the contrary, the y^{ds66} allele, which contains promoter sequences of the putative insertion gene, fails to mediate the repressive effect of *mod(mdg4)* mutations on the bristle enhancer, suggesting that these sequences interfere with the silencing properties of su(Hw) in the absence of mod(mdg4) protein.

The mechanisms by which the su(Hw) insulator affects enhancer-promoter interactions might be explained by a number of alternative possibilities, including interference with looping or tracking of enhancer-bound transcription factors, changes in the adjacent chromatin, or sequestration to particular nuclear compartments unfavorable to transcription. These effects can be overcome by the insertion of large DNA sequences between the su(Hw) insulator and the promoter of the *yellow* gene. Further analyses of the precise structure and nature of these sequences will shed light on the mechanisms whereby the su(Hw) protein affects enhancer function.

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