The P-Ph Protein-Mediated Repression of yellow Expression Depends on Different cis- and trans-Factors in Drosophila melanogaster

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ABSTRACT

The ph allele of Drosophila melanogaster encodes a chimeric P-Ph protein that contains the DNA-binding domain of the P-element transposase and the Ph protein lacking 12 amino-terminal amino acids. It has been shown that the P-Ph protein is responsible for the formation of a repressive complex on P elements inserted at the yellow locus. Here we demonstrate that an enhancer element can suppress the P-Ph-mediated inhibition of yellow transcription. However, an increase of P-element copy number at the yellow locus overcomes the enhancer effect. The mobilization of P-element transposition induced the appearance with a high frequency of Su(y) mutations that partially or completely suppressed the inhibitory effect of ph

expression on yellow expression. The Su(y) mutations were localized at different sites on chromosomes. One strong Su(y) mutation, sn

transcribed noncoding region of the singed locus. The Su(y) mutations resulted in a high level of transcription of the 1.2-kb P element that contained the sequences encoding one DNA-binding and two protein-protein interaction domains of the transposase. The effect of Su(y) mutations can be explained by the competition between the truncated transposase encoded by a 1.2-kb P element and the P-Ph protein for binding sites on P-element insertions.

T HE polyheterotic (ph) gene is a member of the group consisting of at least 20 genes with similar functions that are required for normal segmental specification in Drosophila and referred to as the Polycomb group (Pc-G) genes (Jurgens 1985). The Pc-G genes act in normal development as repressors of BX-C and ANT-C genes (Struehl 1981; Dura et al. 1985; Dura and Ingram 1988). Several Pc-G members were found to be associated in a multigene complex (Franke et al. 1992; Rastelli et al. 1993; Muller 1995; Platero et al. 1996; Strutt and Paro 1997). Cis-regulatory elements necessary for maintaining the repressed state of homeotic genes have been identified (Muller and Bienz 1991; Simon et al. 1993; Chan et al. 1994; Chiang et al. 1995) and designated as Pc-G response elements (PREs; Simon et al. 1993).

We have discovered a new P-element-induced mutation in the polyheterotic (ph) gene, ph

which represses yellow expression in the yellow alleles containing P-element insertions (Belenkaya et al. 1998). The ph

mutation is induced by a 1.2-kb P-element insertion into the 5′ transcribed noncoding region of the ph gene. The ph

allele produces a chimeric protein, designated as P-Ph, that contains the DNA-binding domain of the P element and the Ph protein lacking 12 amino-terminal amino acids. The P-Ph chimeric protein can bind to the P-element sequences and recruit other Pc-G proteins, leading to the formation of a repressive complex.

Previously we described a family of highly unstable mutations induced by chimeric mobile elements located 69 bp upstream to the yellow transcription start site (Georgiev et al. 1997). They appeared against the background of the y allele caused by an insertion of gypsy at −700 bp position (Geyer et al. 1986; Parikh and Corces 1986). The region of gypsy responsible for its mutagenic effect, su(Hw)-binding region, has the properties of an insulator: only yellow enhancers responsible for the body and wing pigmentation located distally from the promoter are affected, whereas all other tissues show the wild-type coloration (Geyer and Corces 1987, 1992). Insertions inducing highly unstable y mutations consist of two identical copies of a defective P element, 1.2 kb long, and some genomic sequences located between them. One such insertion, present in the y

allele, contains a 2.9-kb genomic sequence that includes an enhancer-like element activating yellow expression in the body cuticle and wing blade (Georgiev et al. 1997). A large number of derivative alleles with deletions in the internal part of the chimeric element or with P-element rearrangements can be obtained from the y

allele.

In this article, we have characterized the interactions of the ph

mutation with the y

allele and its derivatives. The inhibiting effect of ph

expression can be suppressed either by the enhancer element located in
the chimeric $y^{+1}$ insertion or by the yellow wing and body enhancers. However, an increase of P-element copy number either in cis or in trans strongly elevates the ph$^-$-mediated repression that overcomes the effect of the enhancers. We have also studied the effect of ph$^-$ on P-element-induced mutations in the singal locus, which is expressed at the same middle pupal stage as yellow during the formation of adult bristles and hairs, and in the female germline during oogenesis (Paterson and O'Hare 1991). Unexpectedly, the ph$^-$ mutation does not influence the phenotype of sn mutations induced even by double P-element insertions.

During this work, we discovered a number of P-element-induced mutations designated as $Su(y)$ that partially or completely suppress the inhibitory effect of ph$^-$ on the $y^{+1}$ allele and its derivatives. The effect of $Su(y)$ mutations results in a high level of P-element expression. The truncated transposase competes with the chimeric P-Ph protein for the same binding sites on P elements.

**MATERIALS AND METHODS**

**Drosophila strains:** All flies were maintained at 25°C in a standard yeast medium. Genetic symbols of the y alleles and the origin of the $y^{+1}$ allele and some of its derivatives were described in other articles (Georgiev et al. 1992, 1997). The w;SbP[y$^{+1}$]v (1996) e TM6,estock, providing a stable source of transposase (Robertson et al. 1988), was obtained from the Bloomington Stock Center (Bloomington, IN). Hereafter P[y$^{+1}$]v (1996) is referred to as $y^{+1}$. The rest of the Drosophila melanogaster strains and mutations are described in Lindsay and Zimm (1992). The origin and structure of the ph$^-$ strain are described in another article (Belenkaya et al. 1998).

**Genetic crosses:** To induce mutagenesis, y$^{+1}$ ph$^-$ females were crossed to w;Sb$^{v2-3}$ e TM6,shmates to produce dysgenic males of the $y^{+1}$ph$^-$/Y; Sb $^{v2-3}$ e TM6,sh genotype. In each vial, $y^{+1}$shmales were mated to 10–12 C(1)RM,Y females. The F$\_1$ progeny was analyzed for mutagenesis. All males with a new phenotype were individually mated to virgin C(1)RM,Y females and their phenotype was examined in the next generation. Determination of the yellow phenotype was performed as previously described (Georgiev et al. 1997).

Combinations of ph$^-$ with different y alleles were obtained according to the following scheme:

- F$\_1$: $\delta y^{+1}ph^+ w/Y, y^{+1}ph^+ w^+ \times \delta y^+/Y$, where y$^+$ is any y allele used;
- F$\_2$: $\delta y^{+1}ph^+ w/Y \times y^{+1}ph^+ w/Y$;
- F$\_3$: Selection of y$^{+1}ph^+ w$ males (sc$^+$ phenotype and orange eyes).

The introduction of ph$^-$ was confirmed by Southern blot hybridization with labeled probes from the ph gene. The w$^+$ (1–1.5) mutation was used as the closest marker for the ph gene (1–0.5).

The removal of ph$^-$ from a X chromosome with a y$^+$ allele was performed according to the following scheme:

- F$\_1$: y$^{+1}sc-ph^+ w/Y \times \delta y^+/Y$;
- F$\_2$: Selection of y$^{+1}ph^+ w$ males (sc$^+$ phenotype and orange eyes).

The removal of ph$^-$ was confirmed by Southern blot hybridization.

Combinations of autosomal $Su(y)$ mutations with y$^{+1}$ ph$^-$ were obtained and analyzed according to the following scheme: F$\_1$: $\delta y^{+1}ph^+ v F4 \times \delta y^{+1}ph^+ v; Su(y)/CyO or Su(y)/TM3,5;ab$ analysis of the y phenotype in y$^{+1}ph^+ v Y; Su(y)^+/+ males.

To study the eye phenotype on the ph$^-$ background we constructed the yph$^-$" line. The su(Hw)/su(Hw)$^-$ mutations in the su(Hw) gene were combined with the y$^{+1}$ ph$^-$ mutations as described previously (Georgiev and Kozychina 1996). The su(Hw)$^-$ mutation is deletion of the su(Hw) gene, whereas su(Hw)$^+$ is a strong mutation induced by jokey insertion in the first intron of the su(Hw) gene (Harrison et al. 1993).

**DNA manipulations:** DNA from adult flies was isolated using the protocol described in Ashburner (1989). The genomic DNA was digested with restriction enzymes according to the supplier's instructions and separated in standard agarose gels (Sambrook et al. 1989). The DNA was transferred to Hybond membranes and probed according to the supplier's instructions. The DNA fragments used as the probes were separated in agarose gels and purified using Gene Clean II (BIOsion, Vista, CA) according to the supplier's instructions.

The genomic DNAs were subjected to PCR to amplify sequences from the derivative alleles (Saiki et al. 1985; Mullis and Faloona 1987). The primers used in DNA amplification were as follows: ATGACATTCTATGCACGATCTCC (y-1, 2409–2432 bp), CAGCGAAGGTGATGTCGACTC (y-2, 2606–2628 bp), TCTGTAGACCTGCGGCTTAC (y-3, 2898–2977 bp), and ACTCTTACTTACTCCAGCCAC (y-4, 3293–3270 bp) for the y gene according to sequences of the y gene presented in Geyer et al. (1986); CGCTTGAGAACCTGAAAGGCC (c-1) and ACTGCGGTCTTCAAGCTCTACC (c-2) for the internal genomic insertion of the y$^{+1}$ allele; AAGCCTACGCTGTGTTCCATAG (sn-1, 857–898 bp) and ACACCCCTACTTCCGCAAATCG (sn-2, 1632–1609 bp) for the singed gene according to sequences of the y gene presented in Paterson and O'Hare (1991); and CTCTCAACCGCCACGGACTG (p-1, 89–66 bp), CGTCGGCACAACCTTCTCTCTC (p-2, 108–85 bp), CTCATACGCACACTGAATTACT (p-3, 2802–2824 bp), and ATACGTAAATGCTGTCTCTTG (p-4, 2853–2875 bp) for the element according to sequences of the P element presented in O'Hare and Rubin (1983). The amplifications were performed with a program including 1 cycle of 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 64°C, 68°C (the temperature depended on the primer), and 2 min at 72°C, and the last, 4 min at 72°C.

The PCR products were directly sequenced using a Sequenase II DNA sequencing kit for PCR product (Amersham, Arlington Heights, IL) according to manufacturer's instructions.

**Northern blot hybridization:** Total RNA was isolated by homogenization in 4 m guanidine isothiocyanate, 0.2% N-lauryl sarcosine, 150 mm mercaptoethanol, 12.5 mm EDTA, and 50 mm tris-hydrochloride, pH 7.5, with subsequent phenol extraction and ethanol precipitation (Parkhurst et al. 1988). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT) cellulose. Northern blot analysis was carried out as described by Parkhurst et al. (1988).

**Construction of P[Su($\omega$), $\Delta$yellow] and P-element transformation:** The structure and expression of the yellow gene were described earlier (Geyer et al. 1986; Geyer and Corces 1987). The 10-kb fragment including the yellow gene and its regulatory 2-kb region was provided by Dr. P. Geyer. The regulatory Sal/BamHI yellow region (3 kb) was subcloned in pGEM7 vector. A plasmid with two ligated Xhol-Sacl fragments was selected by PCR analysis. After that, the double Xhol-
Figure 1.—Schematic presentation of the derivatives obtained from the y\textsuperscript{1A} allele and their interaction with the ph\textsuperscript{11} and su(Hw) mutations. The directions of transcription of the yellow gene and of the 1A gene are indicated by arrows. The P-element sequences and their size are shown by boxes. The P-element orientation is indicated by an arrow. 1A-En is the enhancer located in the chimeric element. Thin lines show the deletion in the 2.9-kb genomic sequence of chimeric mobile element in the y\textsuperscript{102} allele. Sequences remaining after P-element excision are indicated below a corresponding P element. The structure of y\textsuperscript{2} is shown in the lower part of the diagram. Restriction enzymes are as follows: B, BamHI; H, HindIII; P, PstI; R, EcoRI; X, XhoI. The total number of black and white circles indicates the level of pigmentation in ph\textsuperscript{+} flies in 1, body; 2, wings; 3, thoracic bristles; 4, leg bristles; 5, wing bristles; and 6, abdominal bristles. The number of white circles shows the level of pigmentation in flies with the ph\textsuperscript{11} mutation. Thus the number of black circles shows the effect of the ph\textsuperscript{11} mutation. One circle represents one point as in Table 1. The effect of the ph\textsuperscript{11} mutation was also studied on the su(Hw)\textsuperscript{-} and su(Hw)\textsuperscript{+} backgrounds. The su(Hw)\textsuperscript{-} background corresponds to the trans-heterozygous su(Hw)\textsuperscript{+/}su(Hw)\textsuperscript{2}.

Scal fragment was cut out, blunt ligated, and cloned in the regulatory Sall/BamHI yellow region (3 kb) in the KpnI site (Figure 1). One of the obtained clones was sequenced to confirm that no unwanted changes in the yellow sequence had taken place. The 3-kb yellow fragment cut out by BamHI and Xbal was subsequently cloned in the BamHI and Xbal sites of the CaSpeR3 plasmid with a 5-kb coding region of the yellow gene (Figure 1). The new construct was denoted as P\{yellow\textsuperscript{+}, ∆P-yellow\} where w\textsuperscript{-} indicates a minigene included into the vector and ∆P-yellow indicates a complete yellow gene with an insertion of a small part of the P element. The control construct P\{yellow\textsuperscript{+}\} has an unchanged structure of yellow sequences. This construct was obtained by direct cloning of the 10-kb fragment including the yellow gene in the CaSpeR3 plasmid.

DNAs of the P\{yellow\textsuperscript{+}, ∆P-yellow\} and P\{yellow\textsuperscript{+}, yellow\textsuperscript{+}\} constructs and of a P element with a defective inverted repeat used as a transposable source, P25.7\textsuperscript{wc} (Kares and Rubin 1984), were injected into y\textsuperscript{-ac} w\textsuperscript{118} preblastoderm embryos as described previously (Rubin and Spradling 1982; Spradling and Rubin 1982). Survivors were crossed to y\textsuperscript{-ac} w\textsuperscript{118} flies and transgenic flies were separated on the basis of eye color. Chromosome assignments of the various transgene insertions were made by crossing the transformants with y\textsuperscript{-ac} w\textsuperscript{118} balancer stock containing dominant markers: In(2R)\textsuperscript{Cyo} for the second chromosome and In(3L)\textsuperscript{RIT} M 3,5b for the third chromosome. Chromosomal insertions of P\{yellow\textsuperscript{+}, ∆P-yellow\} and P\{yellow\textsuperscript{+}, yellow\textsuperscript{+}\} were made homozygous and their copy number was determined by Southern blot analysis using the yellow and white sequences as probes.

RESULTS

Cis-elements influencing the repression effect of the ph\textsuperscript{11} mutation on yellow expression: In a previous article (Belenkaya et al. 1998), we found that the ph\textsuperscript{11} mutation completely inhibited yellow expression in y\textsuperscript{2} alleles induced by two copies of P element inserted 69 bp upstream of the yellow promoter and mildly inhibited it in y\textsuperscript{2} alleles induced by a single P element copy at the same position. In addition, all described y alleles contained a gypsy insertion at ~700 bp position. The su(Hw) insulator located in the 5' regulatory region of gypsy blocks the yellow wing blade and body enhancers. As a result, the y alleles display y\textsuperscript{-} phenotype (yellow color of body cuticle and wings).

In this article, we studied the y\textsuperscript{1A} allele (Georgiev et al. 1997) that contains, in addition to a pair of 1.2-kb P elements, a duplicated 2.9-kb genomic sequence inserted between them (Figure 1). The P elements were
designated as P1 and P2 depending on whether they were located distally or proximally with respect to the yellow promoter. The 2.9-kb genomic sequence originated from the 1A region of the X chromosome. An efficiently transcribed putative gene designated as the 1A gene was found in the middle of the 2.9-kb sequence (Figure 1). $\gamma^{+12}$ flies have wild-type level pigmentation in body cuticle and wing blade in contrast to flies with the original $\gamma^-$ allele, in which the body and wing enhancers are blocked by $su(Hw)$ insulators. A number of derivatives with $\gamma^-$ phenotype were obtained from the $\gamma^{+12}$. All of them had deletions of various size in the 2.9-kb genomic sequence of chimeric mobile element (Georgiev et al. 1997; I. Birukova and P. Georgiev, unpublished results). The mapping of these deletions allowed us to localize the enhancer element between the 1A gene and P2 element (Figure 1).

The $ph^{11}$ mutation only partially repressed yellow transcription in the $\gamma^{+12}$ allele: the pigmentation of the body, wings, thoracic, and leg bristles was decreased (Figure 1). Thus, the presence of the genomic insertion possessing the 1A enhancer reduces the effect of the P-Ph protein.

To further analyze the role of different cis-regulatory elements in formation of repressive complex, we studied the $\gamma^{+12}$ derivatives obtained after P-element mobilization in the $\gamma^{+12} ph^{11}$ and $\gamma^{+12}$ strains. The structure of insertions in some of these derivatives was described in a previous article (Georgiev et al. 1997), while for new $\gamma$ alleles, it was determined using Southern blot analysis and the PCR cloning and sequencing technique. The presence of nonmodified P element in the $ph^{11}$ allele was verified by Southern blot analysis as described by Belenkaya et al. (1998). All derivatives may be divided for several classes because of their structure and the interaction with $ph^{11}$. To simplify the presentation, we described here only one representative $\gamma$ allele for each class of derivatives, although several independently obtained $\gamma$ alleles with the identical or similar structure have been analyzed in each case.

The $\gamma^{+102}$ allele was obtained as a result of 1A enhancer deletion (Georgiev et al. 1997). The combination of $\gamma^{+102}$ with the $ph^{11}$ mutation led to a complete inhibition of yellow expression (Figure 1). Thus, the 1A enhancer is responsible for the reduction of the P-Ph protein effect.

The $\gamma^{+127}$ allele was generated by excision of P1 element. PCR cloning and sequencing showed that only 15–17 bp from each terminus were retained (Figure 1). The $ph^{11}$ mutation failed to influence the phenotype of $\gamma^{+127}$ flies, suggesting that the 1A enhancer completely overcomes $ph^{11}$-mediated repression, if only one P element is present at the yellow locus.

The $\gamma^{+}$ alleles associated with duplication of any P element were characterized by an almost complete inactivation of yellow expression by the $ph^{11}$ mutation (Figure 1). The $\gamma^{+sk11}$ allele was associated with duplication of the P1 element in the head-to-head orientation (Figure 1). Another $\gamma$ allele, $\gamma^{+sk12}$, had P2-element duplication also in the head-to-head orientation. Both $\gamma^{+}$ alleles had the same strong mutant phenotype in combination with the $ph^{11}$ mutation. Thus, only the number of P-element copies at the yellow locus was important for the enhancement of the effect of the $ph^{11}$ mutation, but not their localization in the chimeric element.

The $\gamma^{+12}$ allele was induced by an inversion of the central 2.9-kb region without P-element deletion (Georgiev et al. 1997). $\gamma^{+12}$ flies had the wild-type level of pigmentation indicating the 1A enhancer to act in an orientation-independent fashion. However, the $ph^{11}$ mutation had a stronger effect on the $\gamma^{+12}$ alleles than on the original $\gamma^{+12}$ allele (Figure 1). Thus, the orientation of the 2.9-kb insertion influences the level of $ph^{11}$-mediated repression. Possibly, the insertion of the 1A promoter between the 1A enhancer and the yellow promoter facilitates inhibition by the P-Ph protein of the 1A enhancer action on yellow transcription. Another explanation is that the inversion brings a distance-dependent negative element cooperating with $ph^{11}$ into close vicinity to the yellow promoter. However, the $\gamma^{+12}$ derivative, which had, in addition to the P2 element, a 410-bp sequence just from the distal part of the 2.9-kb insertion (Figure 1), was affected by $ph^{11}$ to the same extent as $\gamma^{+}$ alleles with an insertion of the P2 element alone (Figures 2 and 3).

To further study the dependence of $ph^{11}$ repression on the orientation of the chimeric element, we mobilized P elements in the $\gamma^{+12} ph^{11}$ strain. As a result, we obtained a $\gamma^{+12} ph^{11}$ derivative characterized by a variegated pigmentation of bristles in combination with $ph^{11}$ (Figure 1). The $\gamma^{+12}$ allele was generated by an excision of P2 element, leaving only 14 and 18 bp of the terminal repeats. Thus, the $ph^{11}$ mutation can inhibit yellow expression in the presence of just a single distal P element located at a 3-kb distance from the yellow promoter if an inversion of the 2.9-kb sequence takes place.

The upstream region of the yellow gene contains the yellow wing and body enhancers, which are blocked due to the presence of the $su(Hw)$ insulator (Figure 2). To study the role of yellow enhancers in the $ph^{11}$-mediated repression, we inactivated the $su(Hw)$ insulator, crossing the combination of $\gamma$ and $ph^{11}$ mutations into $su(Hw)^-$ background. The $su(Hw)^{+/y} su(Hw)^{+11}$ transheterozygote completely suppressed the mutant $\gamma$ phenotype of flies carrying the combination of the $ph^{11}$ mutation with either $\gamma^{+12}$ or $\gamma^{+12}$ mutation (Figure 1). Thus, the yellow body and wing enhancers are also able to suppress a weak $ph^{11}$-mediated repression.

The $su(Hw)^{+/y} su(Hw)^{+11}$ transheterozygote partially suppressed the $ph^{11}$-mediated repression in the $\gamma^{+sk11}$, $\gamma^{+sk11}$, and $\gamma^{+12}$ alleles. This result shows that 1A and yellow enhancers cooperatively protect yellow expression from the $ph^{11}$ repression. However, the yellow enhancers fail to influence strong repression in the $\gamma^{+102} ph^{11}$; $su(Hw)^{+/y}$.
su(H w)\textsuperscript{2} flies (Figure 1). Thus, the yellow enhancers have weaker effect on the ph\textsuperscript{p1}-mediated inhibition of transcription than the 1A enhancer in the y\textsuperscript{y1s42} allele.

**The P-element sequences responsible for ph\textsuperscript{p1}-mediated repression:** In an earlier article (Belenkaya et al. 1998), we demonstrated the absence of ph\textsuperscript{p1} effect if the P-element deletion led to the loss of transposase-binding sites. Here we have further studied the role of different P-element sequences. From this, the derivatives of y\textsuperscript{y2s14} and y\textsuperscript{y1s42} alleles generated by partial deletions of the P elements were characterized. The breakpoints of the deletions in the P elements were cloned by PCR and sequenced. The effect of the ph\textsuperscript{p1} mutation was studied in y\textsuperscript{y}/ph\textsuperscript{p1}/Y males and in y\textsuperscript{y}/y ph\textsuperscript{p1} heterozygous females.

As was shown previously, the ph\textsuperscript{p1} mutation mildly repressed yellow expression in the y\textsuperscript{y2s14} allele containing a single P-element copy (Belenkaya et al. 1998). Its derivative, y\textsuperscript{y2s34} allele, was induced by a P element with an internal deletion. The breakpoints of the deletion were mapped at distances of 767 bp from the 5' terminus and 93 bp from the 3' terminus; i.e., both termini contained transposase-binding regions, but the 11-bp inverted repeat at the 3' terminus was deleted (Figure 3). The ph\textsuperscript{p1} mutation had no visible effect on the yellow expression in combination with y\textsuperscript{y2s34}. The suppression of the ph\textsuperscript{p1} effect may be explained by the previous observation that the 11-bp inverted repeat serves as an additional binding site for a truncated transposase (Lee et al. 1996).

Another derivative, y\textsuperscript{y2s34} allele, which had only 41 bp of the 3' end and 852 bp of the 5' end, was affected by ph\textsuperscript{p1}: the notum and leg bristles acquired a variegated mutant phenotype (Figure 3). The P element in the y\textsuperscript{y2s34} allele had a deletion of 3' transposase-binding sites, but it differed from the y\textsuperscript{y2s14} allele by the presence of a part of the central region of the defective P element between 767 and 852 bp. The latter included the breakpoint of the deletion and the filler sequence TAGCTACAAA, suggesting the presence of additional binding sites for the chimeric P-Ph protein in this region.

To identify the role of the central region of the P element in the ph\textsuperscript{p1} repression, we prepared the P \{w\textsuperscript{y}; \Delta P-yellow\} construct containing two 179-bp XhoI-ScaI restriction fragments of the 1.2-kb P element (the fragment between 730 and 909 bp of the latter) at the position of -340 bp from the yellow transcription start site (Figure 2). Seven independent transformants were obtained, possessing a single insertion as confirmed by Southern blot analysis. All of them displayed wild-type levels of yellow expression. The combination of the ph\textsuperscript{p1} mutation with the P \{w\textsuperscript{y}; \Delta P-yellow\} construct in five cases led to mosaic pigmentation of bristles, while the pigmentation of the body and wings was not changed (data not shown). The level of repression was the same for
heterozygous and homozygous states of the P \(w^+, \Delta P\)-yellow transposon, which contained the unmodified yellow gene. Nine transgenic lines carrying P \(w^+, \Delta P\)-yellow transposon were obtained. The y phenotype of flies from these lines was not changed on a ph\(^{p1}\) background. This result confirms the role of the central Xhol-ScaI region of the 1.2-kb P element in the ph\(^{p1}\)-mediated repression of the yellow expression.

We also compared eye pigmentation in either the presence or absence of the ph\(^{p1}\) mutation. The miniwhite gene is very sensitive to repression by Pc-G complex. For example, a 661-bp PRE core fragment from the Ubx gene, inserted in a CaSpeR3 vector, generally causes variegated miniwhite gene expression in 50–60% of the lines (Chan et al. 1994). We found that only one of six lines carrying P \(w^+, \Delta P\)-yellow transposon in heterozygous state displayed a weak decrease of eye pigmentation after cross to a ph\(^{p1}\)w background. In the homozygous state of the P \(w^+, \Delta P\)-yellow transposon, flies from four of six lines showed decrease of eye pigmentation and a weak variegation characteristic for Pc-G repression of white expression (data not shown). Thus, the ends of P elements in the presence of ph\(^{p1}\) have only a weak repression potential compared to natural PREs.

As shown previously (Belenkaya et al. 1998), the ph\(^{p1}\) mutation mildly repressed yellow expression in the y\(^{314}\) and y\(^{326}\) alleles containing a single P-element copy, whereas it completely inhibited yellow expression in the y\(^{321}\) allele induced by an insertion of two P-element copies in the head-to-head orientation (Figures 2 and 3). The y\(^{321}\) allele contains the P1 element with conserved ends and the P2 element with a deletion of 5'-terminal 82 bp. This deletion includes the 5'-terminal binding sites for the P-element transposase or the P-Ph protein. To find a minimal region of the P 2 element that was sufficient for strong enhancement of the ph\(^{p1}\) repressive effect, we analyzed the structure of two y\(^{326}\) mutations, derivatives of y\(^{314}\), which in combination with ph\(^{p1}\) led to a complete inactivation of yellow expression. Both y alleles contained the unchanged P1 element. The y\(^{325}\) allele contained only 39 bp of the 5’ terminal sequence and 349 bp of the 3’ terminus of the P 2 element. The y\(^{326}\) allele contained just 153 bp of the 3’ terminal sequence of the P 2 element. However the ph\(^{p1}\) mutation still completely repressed the yellow expression in these alleles. Thus, the addition of the P-element transposase binding sites and the 11-bp inverted repeat to the single P element is sufficient for strong enhancement of the ph\(^{p1}\)-mediated repression.

To make the difference between strong and weak ph\(^{p1}\)-mediated repression more clear, we introduced the combinations of ph\(^{p1}\) with the y mutations into a su\((Hw)^+\) background (Figure 3). The su\((Hw)^+\)/su\((Hw)^+\) transheterozygote completely suppressed the mutant yellow phenotype of flies carrying the combination of the ph\(^{p1}\) mutation with either y\(^{324}\) or y\(^{326}\) mutation induced by single P-element insertion. Thus, the yellow body and wing enhancers are able to suppress a weak ph\(^{p1}\)-mediated repression. On the contrary, the su\((Hw)^+\)/su\((Hw)^+\) transheterozygote only weakly influences the phenotype of flies carrying the ph\(^{p1}\) mutation with either y\(^{321}\), y\(^{326}\), or y\(^{325}\) alleles. Thus, the 3’ sequences of the P element can strongly enhance the ph\(^{p1}\)-mediated repression.

**Pairing-dependent repression of yellow expression mediated by ph\(^{p1}\):** The repressive effect of the ph\(^{p1}\) muta-
PhP1-mediated trans repression of transcription in females heterozygous for y alleles

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The figures show the level of pigmentation estimated visually in 3- to 5-day-old females developing at 25°C. The level of pigmentation was evaluated on a scale from 0 to 5. Zero is the pigmentation of y* flies. Two numbers indicate the variable pigmentation of bristles. Th, thoracic; L, leg; W, wing; Ab, abdominal.

Thus, a single P-element copy failed to induce a strong pairing-dependent repression by the phP1 mutation. In contrast, the y³⁷, y³⁵¹, and y⁳⁵ alleles induced a strong pairing-dependent repression (Table 1). These y³ alleles were induced either by two P elements or by the P2 element and the 3' terminus of the P1 element (Figures 2 and 3). However, the effect of phP1 was weaker in heterozygous females with the y²⁶ allele. The y²⁶ allele differed from the y²⁵ allele only by a deletion of the central region of the P1 element, further confirming the role of this part of the 1.2-kb P element in the phP1-mediated repression of yellow transcription.

**Su(y) mutations**: In the progeny obtained after P-element mobilization in y¹¹ phP1 flies, we could frequently observe the appearance of autosomal suppressors of phP1-mediated repression. These mutations, designated as Su(y), were widely spread on the X, second, and third chromosomes and displayed different levels of the suppression effect. Eleven Su(y) mutations were lethal in the homozygous state, 9 possessed normal or decreased viability, and 2 were associated with female sterility. One of the Su(y) mutations appeared simultaneously with a mutation in the ebony (e) locus, and the recombination analysis showed them to be one and the same mutation. Another strong Su(y) mutation was obtained in the singed locus (sn*).

For further study, we selected three Su(y) alleles, Su(y)⁴ (third chromosome), Su(y)⁵ (second chromosome), and sn⁶. To compare the levels of suppression,
The most plausible explanation was that Su(y) mutations were induced by P-element insertions into regulatory or transcribed regions of different genes that led to a high level of transcription of the defective P elements and to the accumulation of truncated transposase that might suppress the mutant phenotype of the y alleles competing with the P-Ph protein for binding sites. To test this hypothesis, we performed a Northern blot analysis of mRNA from the Su(y) and control strains using as a probe the P-element HindIII-Xhol fragment that included the sequences encoding the DNA-binding domain of transposase (Andrews and Glover 1995; Lee et al. 1996). A high level of mRNA hybridizing with the P-element sequences was detected only in the Su(y) strains (Figure 4). The highest level of P-element transcripts was found in the Su(y)P31 strain at the larval, pupal, and adult stages of development. The Su(y)P22 strain had a lower but still rather high level of P-element transcripts at all tested stages of development. In the sn¹ allele, a high level of P-element transcripts was detected only at the early pupal stage of development, when the singed gene was actively transcribed (Paterson and O’Hare 1991).

Molecular study of the sn¹ allele: To gain a better insight into the mechanism of Su(y) action, we used for molecular analysis the sn¹ mutation in the well-characterized singed locus (Roihal et al. 1988; Paterson and O’Hare 1991). The sn¹ mutation had a strong mutant phenotype. Southern blot hybridization revealed an ~1.0-kb insertion localized between the EcoRI and SalI restriction sites of the DNA segment (Figure 5). This insertion was within the 700-bp singed “hot spot” region (Hawley et al. 1988; Roihal et al. 1988). For cloning of the P-element insertion, the oligonucleotides were designed from both sides of the insertion (Figure 5). The PCR-amplified fragment was directly sequenced. The sn¹ insertion was shown to be a 1180-bp internally deleted P element with a structure identical to that described for the P element responsible for superunstable y alleles and ph¹ mutation. Based on the published sequences of a full-length P element (O’Hare and Rubin 1983), this sn¹ P element contained an internal deletion from 830 to 2560 bp and a filler sequence TAGCTACAAA at the break point. Interestingly, as in the case of highly unstable y mutations, the P-element insertion at the singed locus was associated with a deletion: 4 bp (CGCG) at the target site were lost, and no 8 bp duplication typical of P-element insertions was observed. The P element in the sn¹ allele is localized in the transcribed but not translated part of the singed gene ~80 bp downstream from the signal of transcription initiation (Paterson and O’Hare 1991; Burke and Kadowaga 1996).

To prove the responsibility of sn¹ for the Su(y) effect, we studied revertants induced by crossing with Δ2-3. Five sn⁴ revertants were obtained and shown by Southern blot hybridization to lack the P element. As expected, the sn⁴ revertants, sn⁺P¹ and sn⁺P², simultane-
D. melanogaster plays a role in both somatic and germ duplication is flanked by two identical copies of a deletion in the Sne mutation. The deletion in the Sne gene is important for realization of the P element phenotype, which is encoded by the P element sequences and leads to P-element silencing. The deletion is shown in the lower part of the figure. The duplicated eight nucleotides of the host DNA are underlined. The deleted cgcc nucleotides are shown by lowercase letters. The primers used for PCR cloning are shown by arrowheads.

Figure 5.—Structure of sn alleles. Start and direction of singed transcription is indicated by an arrow. Insertions of P elements responsible for different sn alleles are represented by triangles. The P-element sequences and their size are shown by boxes. The orientation of P elements is indicated by arrows. The sequence of the region of P-element insertions is shown in the lower part of the figure. The duplicated eight nucleotides of the host DNA are underlined. The deleted cgcc nucleotides are shown by lowercase letters. The primers used for PCR cloning are shown by arrowheads.

additionally lost the ability to suppress the mutant phenotype of the yuiph+ combination (Figure 5). We also studied two sn alleles, snw13 and snw14, obtained from sn as a result of P-element duplication in the head-to-head orientation, as in the case of the well-known sn mutation (Roiha et al. 1988). The sn alleles only slightly suppressed the yuiph+ mutant phenotype, possibly due to a low level of P-element transcription (Figure 5).

The P elements in the previously described sn mutation (Engel's 1989) are located in the same region as in the sn+ mutation. The sn allele contains two P elements of 1.15 and 0.95 kb inserted at the same site in the inverse orientation. Excision of the 0.95-kb P element gives rise to sn+, and excision of the 1.15-kb P element, to sn+ (Roiha et al. 1988). The sn+ and sn+ mutations did not influence the effect of ph+ 1. Only the sn+ allele had a weak suppressive effect (Figure 5). The P elements in sn+ and sn+ have the same orientation and are inserted at a distance of 13 bp from each other (Figure 5). The only difference between the P elements is the deletion in P element inserted in the sn+ allele of the second region important for the protein-protein interaction that enhances DNA binding (Lee et al. 1996). This result shows that a strong DNA-binding ability is important for realization of the Su(y) effect.

The ph+ mutation does not affect singed expression in P-element-induced sn mutations: The singed gene of D. melanogaster plays a role in both somatic and germ cells (Lindsley and Zimm 1992). The bristles and hairs are shortened or twisted and gnarled in sn mutants. In addition to this phenotype, many strong sn mutations lead to female sterility (Lindsley and Zimm 1992).

The P element in the above described sn mutations was inserted at a distance of <50 bp from the core region of the downstream promoter element (DPE) of the singed gene (Paterson and O'Hare 1991; Burke and Kadosh 1996). However, the ph+ mutation did not increase the visible mutant phenotype of the sn mutations induced by one (sn1, sn2, and sn3+) or two (sn+, snw13, and snw14) P elements (data not shown). This result could not be explained by the fact that sn mutations suppressed the ph+ effect, because such a weak suppressor as the sn+ mutation also was not modified by ph+1. The ph+ mutation also did not influence the sn-phenotype in females, suggesting the absence of the pairing effect.

P-element-induced sn mutations did not significantly influence the singed function in gonads: females homozygous for a sn mutation were fertile. Only the snw13 mutation reduced female fertility. The ph+ mutation did not significantly increase the sterility of females with homozygous sn mutations.
for yellow activation in the body cuticle and wing blade (Georgiev et al. 1997) compensating the yellow body and wing enhancers blocked by the su(Hw) insulator. The appearance of a strong enhancer disturbs the repressive effect of the P-Ph protein. Addition of the wing and body enhancers by inactivation of the su(Hw) function further suppresses the silencing effect of the \( \text{ph}^{1i} \) mutation. It has been shown that the assembly of a silencing complex depends both on the strength of a PRE site and on the transcriptional activity of the region involved (Pirrotta 1997). For example, the formation of a Pc-G complex and the binding of GAL4, which activates transcription, are mutually exclusive and the silencing state can be prevented by a strong activation of transcription (Zink and Paro 1995; Cavalli and Paro 1998).

The level of repression induced by \( \text{ph}^{1i} \) depends directly on the number of P elements but not on their localization. The \( \text{ph}^{1i} \)-mediated repression may act at a distance from the yellow promoter. In the \( y^{133} \) allele the P element is located \( \sim 3 \) kb from the yellow promoter and is separated from the latter by the \( 1A \) enhancer. Still in this case, the \( \text{ph}^{1i} \) mutation inhibits yellow transcription. In this respect, the P elements act like PREs that are responsible for the repressive state in the adjacent genome region in a cooperative manner (Orlando and Paro 1993; Pirrotta 1997).

The \( \text{ph}^{1i} \) mutation induces only a weak repression in \( y \) alleles containing a single P-element copy upstream of the yellow gene. Thus, one copy of P element is not sufficient for the formation of a strong repression complex. Addition to a single copy of the 1.2-kb P element of 153-bp 3’-terminal P-element sequences strongly enhances \( \text{ph}^{1i} \)-mediated repression, leading to a complete inactivation of yellow expression. As expected, the deletion of transposable-binding sites or 11-bp inverted repeats reduces the \( \text{ph}^{1i} \)-mediated repression. Unexpectedly, the internal region of the 1.2-kb P element is also important for the \( \text{ph}^{1i} \)-mediated repression that suggests the presence of a potential site(s) for binding of the chimeric P-Ph protein.

PRE-containing transposons often show a dramatic enhancement of silencing when a fly is homozygous for a transposon insertion, indicating that the homology-paired PREs interact to produce a more stable and more repressive Pc-G complex (Fauvarque and Dura 1993; Chan et al. 1994; Kassis 1994). We did not find any pairing-dependent repression when both paired \( y \) alleles contained a single P-element copy inducing a weak or no \( \text{ph}^{1i} \)-mediated repression. A pairing-dependent repression was found only if one of the \( y \) alleles in heterozygous females induced a strong repression complex in the presence of \( \text{ph}^{1i} \).

Truncated P-element transposase similar in structure to the KP protein suppresses the inhibitory effect of the chimeric P-Ph protein on the expression of P-element-induced \( y \) alleles: The inhibitory effect of the \( \text{ph}^{1i} \) mutation can be partially suppressed by Su(y) mutations induced by a P-element insertion into regulatory or coding regions of different genes. The inserted 1.2-kb P element is transcribed at a high level and gives rise to a truncated transposase. For example, in the \( \text{sn}^{1i} \) mutation, the 1.2-kb P element is inserted into the 5’ transcribed noncoding region leading to an enhanced P-element transcription. As shown before, mRNA encoding truncated transposase was also formed as a result of the \( \text{ph}^{1i} \) mutation (Belenkaya et al. 1998) and this may partially decrease its effect.

The 1.2-kb P element has a deletion between 830 and 2560 bp and resembles the previously described KP element (Black et al. 1987). The DNA-binding domain is located within the region of 98 amino-terminal amino acids (Rio 1990; Andrews and Gloor 1995). The KP protein also contains two protein-protein interaction regions, and dimerization of the KP protein is essential for high-affinity DNA binding (Lee et al. 1996). A putative leucine zipper is located between 101 and 122 amino acids of the KP protein (Rio 1990; Andrews and Gloor 1995). The second protein-protein interaction region is present within a segment of 69 carboxy-terminal amino acids of the KP protein, i.e., still in the amino-terminal part of the intact P element (Lee et al. 1996). All these sequences are also present in the protein encoded by the 1.2-kb P element.

The KP protein binds to multiple sites on the P-element termini with a higher affinity than the full-length transposase (Lee et al. 1996). The binding sites include the high-affinity transposase binding sites, an 11-bp transpositional enhancer, and the terminal 31-bp inverted repeats. Both protein-protein interaction regions are important for this binding. The 1.15-kb P element in \( \text{sn}^{1i} \), a derivative of the \( \text{sn}^{1} \) mutation (Roitha et al. 1988; Engel 1989), is inserted in almost the same place and has the same orientation as the P element in \( \text{sn}^{1i} \). The protein product of the 1.15-kb P element contains the DNA-binding domain and a leucine zipper, but not the second protein-protein interaction region (Roitha et al. 1988; Lee et al. 1996). This explains why the \( \text{sn}^{1i} \) mutation strongly suppresses the \( \text{ph}^{1i} \) inhibitory effect, while \( \text{sn}^{1} \) does not. The \( \Delta 2-3 \) construct generating a full length transposase has a very weak suppression effect on the \( \text{ph}^{1i} \) mutation (T. Belenkaya, unpublished results). This may also be explained by its lower affinity to the P-element DNA.

The defective transposase with the DNA-binding domain and two protein-protein interaction domains is most efficient for realization of the suppression effect. Thus, the suppression of \( \text{ph}^{1i} \)-induced inhibition is a result of the competition for binding sites on the P elements between the KP-like protein and the P-Ph protein.

The presence of a strong enhancer in the chimeric element helps the KP-like protein in blocking the assembly of a P-Ph-mediated Pc-G complex on the P element.
However, the Su(y) mutations, even expressing the truncated P-element transposase at high levels at all stages of Drosophila development, fail to affect the ph\(^{R_1}\)-mediated inhibition of \(y^+\) alleles induced by a double P element in the absence of the 1A enhancer. The formation of a strong repression complex prevents binding of the KP protein to transposase-binding sites on the P elements. Thus, the general low level of transcription activity facilitates P-Ph protein binding to DNA and the formation of a repressive complex.

**singed expression is insensitive to the ph\(^{R_1}\) mutation in the P-element-induced mutations**

The singed gene plays a role in somatic cells during the formation of adult bristles and hairs, and in the female germline during oogenesis (Pater son and O’hare 1991). During metamorphosis, a single 3.6-kb RNA is transcribed, but in ovaries two additional transcripts, 3.3- and 3.0-kb RNAs, appear. The mRNAs differ only in the length of the 3’-untranslated region and a single gene product is predicted for all of them. The singed gene contains a downstream promoter element (DPE) that is located only 50 bp upstream from the sites of insertions of P elements in the sn mutations (Pater son and O’hare 1991; Burke and Kadonaga 1996). However, the ph\(^{R_1}\) mutation does not influence the bristle phenotype and female sterility of the sn mutants, even those induced by a double P element. This result may be explained either by a special organization of the singed promoter making it insensitive to the P-Ph protein or by a structure of chromatin in a vicinity of the singed locus.

As we have found here, the miniwhite expression in transposons is weakly sensitive to the ph\(^{R_1}\) mutation that may be explained by a large distance between the white promoter and P-element sequences in the transposon. However the effect of the ph\(^{R_1}\) mutation is not restricted to the yellow gene. In another article (Soldatov et al. 1999), we described the strong ph\(^{R_1}\)-mediated repression of TAF\(_1\)/\(\delta\)y1 expression in the \(\delta\)y1 allele that was generated by P-element insertion at the transcribed non-translated region of the gene.

A relatively weak effect of the ph\(^{R_1}\) mutation in several cases may be explained by the low level of the P-Ph protein produced by the ph\(^{R_1}\) allele. To examine this possibility, we are constructing the transgenic lines producing a large amount of P-Ph protein.

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**LITERATURE CITED**


