P-Element Insertion at the *polyhomeotic* Gene Leads to Formation of a Novel Chimeric Protein That Negatively Regulates *yellow* Gene Expression in *P*-Element-Induced Alleles of *Drosophila melanogaster*

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ABSTRACT

Polyhomeotic is a member of the *Polycomb* group (*Pc*-G) of homeotic repressors. The proteins encoded by the *Pc*-G genes form repressive complexes on the polycomb group response element sites. The ph^{P1} mutation was induced by insertion of a 1.2-kb *P* element into the 5′ transcribed nontranslated region of the proximal *polyhomeotic* gene. The ph^{P1} allele confers no mutant phenotype, but represses transcription of *P*-element-induced alleles at the *yellow* locus. The ph^{P1} allele encodes a chimeric P-PH protein, consisting of the DNA-binding domain of the *P* element and the PH protein lacking 12 amino-terminal amino acids. The P-PH, Polycomb (PC), and Posterior sex combs (PSC) proteins were immunohistochemically detected on polytene chromosomes in the regions of *P*-element insertions.

THE *polyhomeotic* (*ph*) gene is a member of a class of at least 30 genes with similar functions that are required for normal segmental specification in Drosophila and that are 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 (Struhl 1981; Dura *et al.* 1985; Struhl and Akam 1985; Dura and Ingham 1988; Simon *et al.* 1992). The finding of a protein motif common to the Polycomb protein (PC) and the heterochromatin-associated HP1 protein led to the supposition that the PC-G proteins might keep the homeotic genes inactivated by generating heterochromatin-like repressive structures (Gaunt and Singh 1990; Paro 1990; Paro and Hogness 1991). The PC protein was shown to cover large chromosomal domains of the homeotic *bithorax* complex BX-C, when it is in the inactive state (Orlando and Paro 1993; Strutt *et al.* 1997). Several *Pc*-G members were found to be associated in a multiprotein complex (Franke *et al.* 1992; Rastelli *et al.* 1993; 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).

The *ph* gene forms a direct tandem repeat on the *X* chromosome and comprises two genetic and molecular units, one of which can largely compensate for a mutation of the other (Dura *et al.* 1987; Deatrick *et al.* 1991). The PH protein contains a possible zinc finger motif, a serine/threonine-rich region, and glutamine repeats. It has been localized to ~80 sites on polytene chromosomes, and an extensive overlap in the localization of these sites has been found between PH and other PC-G proteins, PC, PSl, SU(Z)2, and PSC (De Camillis *et al.* 1992; Franke *et al.* 1992; Martin and Adler 1993; Rastelli *et al.* 1993; Lonie *et al.* 1994).

In this article, we describe a new *P*-element-induced mutation in the *ph* gene, ph^{P1}, which represses *yellow* (*y*) gene expression in *P*-element-induced *y* alleles. This mutation has been derived via the insertion of a 1.2-kb-defective *P* element to the 5′ transcribed noncoding region of the *ph* gene. The 1.2-kb *P* element encodes a truncated transposase protein that possesses a DNA-binding domain and leucine zipper (Andrews and Gloor 1995). The ph^{P1} allele produces a chimeric protein containing the DNA-binding domain of the *P* element and the PH protein lacking 12 amino-terminal amino acids. Immunostaining of polytene chromosomes of the ph^{P1} strain with antibodies to the PH protein shows new sites of PH protein localization that coincide with *P*-element sites. The Polycomb (PC) and Posterior sex combs (PSC) proteins are also bound to the chromatin at the sites of *P*-element localization. The ph^{P1} mutation has a dominant effect, and the level of *yellow* repression directly correlates with the number of *P*-element copies inserted at the *yellow* locus. The ph^{P1} mutation also mediates the pairing-dependent repressive interaction between different *y* alleles. Thus, in the ph^{P1} background,

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the chimeric P-PH protein binds to *P*-element sequences and recruits other *Pc*-G proteins, leading to the formation of a repressive complex.

MATERIALS AND METHODS

Drosophila strains and genetic crosses: All flies were maintained at 25° in a standard yeast medium. Genetic symbols of the *yellow* alleles and their origin have been previously described (Georgiev *et al.* 1992, 1997). The *w; Sb* P($ry^+\Delta 2$–3)99B *e*/*TM1*, *e* stock providing a stable source of transposase (Robertson *et al.* 1988) was obtained from the Bloomington stock center. The P($ry^+\Delta 2$–3)99B strain is referred to as Δ2–3 for abbreviation. The *Pc*-G mutations used in this study are described in Lindsley and Zimm (1992).

To induce mutagenesis in any *y** allele, *y** females were crossed to *w; Sb* Δ2–3 *e*/*TM1*, *e* males to produce dysgenic males with the *y**/*Y; Sb* Δ2–3 *e*/+ genotype. In each vial, from 2 to 3 *y**/*Y; Sb* Δ2–3 *e*/+ males were mated to 10–12 *C(1)RM,y f* females. The F_2 progeny were analyzed for mutagenesis. All males with a new yellow phenotype were individually mated to virgin *C(1)RM,y f* females and the phenotype of the males was examined in the next generation.

For determination of the yellow phenotype, the levels of pigmentation in different tissues of adult flies were estimated visually in 3- to 5-day-old males and females developing at 25°. In every case 20–50 flies were scored. The pigmentation of six regions of the adult cuticle and its derivative structures, *i.e.*, body, wings, thoracic, leg, wing, and abdomen bristles, was analyzed. The level of pigmentation was measured on a scale from 0 to 5. Flies with previously characterized *y* alleles (Georgiev *et al.* 1992) were used as standards to determine levels of pigmentation, and new combinations of 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 Kozycina 1996). Levels of pigmentation intermediate between y^2 and y^{2PR1} were assigned a value of 2, and levels intermediate between y^{2PR1} and y^+ (Canton-S) were assigned a value of 4.

Combinations of ph^{P1} with different *y* alleles were obtained according to the following scheme:

F_0: ♀ $y^1 sc^{D1} ph^{P1} w^a / y^1 sc^{D1} ph^{P1} w^a$ × ♂ *y**/Y;
F_1: ♀ $y^1 sc^{D1} ph^{P1} w^a / y^*$ × ♂ $y^1 sc^{D1} ph^{P1} w^a$/Y;
F_2: Selection of $y^* ph^{P1} w^a$ males
(sc^+ phenotype and orange eyes).

The introduction of ph^{P1} was confirmed by Southern blot hybridization. The w^a mutation was used as the closest marker for the *ph* gene.

DNA manipulations: DNA from adult flies was isolated using the protocol described in Ashburner (1989). 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 N^+ membrane and probed according to the supplier's instructions. The DNA fragments used as probes were separated in agarose gels and purified using Gene Clean II (BIO 101, Inc., Vista, CA) according to the supplier's instructions.

For preparing genomic libraries, the DNAs of the mutant strains were restricted with *Bam*HI endonuclease and subjected to agarose gel electrophoresis. Bands of the appropriate size were cut from the gel, and the DNA was extracted by electroelution. The DNA was ligated to the arms of the lambda DASH II vector (*Bam*HI) provided by Stratagene (La Jolla, CA). The DNA was packaged in the Gigapack II Gold packaging extract (Stratagene). Plating and screening of the DNA libraries were done according to the standard method (Sambrook *et al.* 1989). Subcloning and purification of plasmid DNA and mapping of restriction sites were performed by standard techniques (Sambrook *et al.* 1989).

Genomic DNAs were subjected to PCR to amplify sequences from the derivative alleles (Saiki *et al.* 1985; Mullis and Faloona 1987). The primers used in DNA amplification were as follows: ACTTCCACTTACCATCACGCCAC (y1), ATGCATTCTATGCACGAGCCTCC (y2), TCTGTGGACCGTGGCGCGGTAAC (y3), and CAGCGAAAGGTGATGTCTGACTC (y4) for the *y* gene; TCGCGTGCACAGGTGCTT (ph1), GTATGTAACGTGGAACGCAAGA (ph2), AGTTGAGTGCGTGCCTTA (ph3), GGGCGTGCCACATCGTAT (ph4), and AAGTGCCTGCAGCCAGCG (ph5) for the *ph* gene. The products of amplification were fractionated by electrophoresis in 1–2% agarose gels in TAE.

DNA sequencing was performed by the dideoxy chain-termination methodology. The PCR products were directly sequenced using a Sequenase II DNA sequencing kit for PCR product (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

RNA manipulations: Total cellular RNA was isolated from Drosophila embryos, larvae, pupae, or adult flies according to Maes and Messens (1992). Poly(A), RNA was selected on oligo(dT)-cellulose columns, and 1.5 μg of poly(A), RNA was loaded per lane of agarose gel. After electrophoresis, RNA was transferred to Hybond-N, membrane (Amersham). The hybridization was performed at 50° in high SDS-formamide buffer (7% SDS, 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 50 mm sodium phosphate, 0.1% sarcosyl) overnight. The ^{32}P-labeled DNA probes were obtained in random priming reaction. Membranes were washed twice in 0.1% SDS, 1× SSC at room temperature for 10 min and in 0.1% SDS, 0.2× SSC at 65° for 20 min, and exposed to Kodak BioMaxMS film with Kodak BioMaxMS intensification screen for 2–4 hr.

The first cDNA strand was synthesized using 0.5 μg of mRNA from ph^{P1} with the AGTTGAGTGCGTGCCTTA (ph3) primer by Superscript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The product was purified in an agarose gel and two-step PCR was performed. For the first step the following primers were used: TCGCGTGCACAGGTGCTT (ph1) and AGTTGAGTGCGTGCCTTA (ph3). Then nested PCR with the GGGCGTGCCACATCGTAT (ph4) and AAGTGCCTGCAGCCAGCG (ph5) nested primer was performed. The PCR products were cloned in the pGEM-T vector (Promega, Madison, WI).

***In situ* hybridization to polytene chromosomes:** For *in situ* hybridization, Drosophila polytene chromosome spreads were prepared from salivary glands of third-instar larvae grown at 17°. Preparation of spreads, fixation, denaturation, and hybridization were done as described by Fauvarque and Dura (1993). Labeling was performed with a[^{3}H]dATP and a [^{3}H]dUTP in a random priming reaction.

Immunostaining of polytene chromosomes: Fixation and squashing of salivary glands and antibody staining were performed as described by Platero *et al.* (1996). The polyclonal antibodies to the PH, PC, and PSC proteins were a gift of Dr. R. Paro. Polyclonal antibodies to PH were diluted 1:500; to PC, 1:20; and to PSC, 1:100 with TBS, 0.05% Tween-20, and 10% goat serum, and added to the slide. Cy3-conjugated anti-rabbit antibodies (1:300, Sigma, St. Louis) were used as secondary antibodies. Incubation with the primary antibodies was carried out for 2 hr at room temperature. A minimum of five slides containing squashes from two glands were examined and the results observed were highly reproducible.

TABLE 1

The effect of ph^{P1} on *P*-induced *y* mutations

	Pigmentation					
y alleles	Body	Wings	Th	L	W	Ab
y^{2}	1 (1)	1 (1)	5 (5)	5 (5)	5 (5)	5 (5)
y^{2s14}, y^{2s20}, y^{2sA3}	1 (1)	1 (1)	5 (1)	5 (2)	5 (4)	5 (4)
y^{76d28}	3 (0)	3 (3)	2 (0)	3 (0)	2 (0)	4 (2)
y^{+s7}, y^{+s8}	5 (4)	5 (5)	5 (1)	5 (3)	5 (5)	5 (5)
y^{2s7}, y^{2s11}	1 (0)	1 (0)	5 (0)	5 (0)	5 (0)	5 (0)
y^{ds62}	2 (0)	2 (0)	5 (0)	5 (0)	5 (0)	5 (0)

Numbers indicate the level of pigmentation of adult flies determined visually under a dissecting microscope. Numbers in parentheses show the effect of the ph^{P1} mutation on the expression of a particular *y* allele. Zero corresponds to the pigmentation of flies carrying a null *y* allele, whereas the wild-type level of pigmentation is represented as 5. Flies with well-characterized *y* alleles were also used as controls for estimating the levels of pigmentation (Georgiev *et al.* 1992). Groups of bristles are abbreviated as follows: Th, thoracic; Ab, abdominal; W, wing; L, leg.

RESULTS

Genetic observations on a modifier of *P*-element-induced mutations: Previously we have described a mutation in the *yellow* locus, y^{2s14} (Georgiev *et al.* 1997), which was induced by the insertion of a defective copy of the *P* element at position −69 bp of the *yellow* gene in the background of the y^{2} mutation, *i.e.*, *gypsy* insertion 700 bp upstream of the *yellow* cap site (Geyer *et al.* 1986; Parkhurst and Corces 1986). Flies carrying y^{2s14} have the same phenotype as the original y^{2} mutation, *i.e.*, yellow color of the body and wings and normal pigmentation of the bristles (Table 1). The inserted *P*-element copy is 1.2-kb long, it contains 829 bp from the 5′ end and 347 bp from the 3′ end (from 2560 to 2907 bp), and the non-*P*-element sequence TAGCTACAAA is inserted in between. Its orientation is opposite to the direction of *yellow* transcription (Georgiev *et al.* 1997).

After mobilization of *P*-element transposition, a derivative of y^{2s14} that was characterized by a mutant yellow color of the thoracic and leg bristles was obtained. Southern blot hybridization showed no difference in the structure of the *yellow* locus between the parental y^{2s14} strain and its derivative. A modifier located on the *X* chromosome may have been responsible for the new mutant phenotype. This modifier was mapped by recombination analysis with the $y^{2}sc^{D1}w^{aG}ct^{6}g$ and $y^{1}z^{1}$ strains to the 0.5- to 0.6-cM region. The modifier has a dominant effect on y^{2s14}/y^{2s14} and $y^{2s14}/y1$ females but does not influence the y^{2}, y^{2s}, y^{td}, and y^{+} alleles induced by mobile elements other than the *P* element (Lindsley and Zimm 1992).

In situ hybridization showed that the modifier strain contained a *P*-element insertion in the 2D (1–0.5) region, where the modifier gene was mapped genetically by recombination. Several revertants of the modifier mutation were obtained in the progeny from a cross with the Δ2–3 strain. Southern blot analysis of DNA from flies of the mutant and revertant strains restricted with *Bam*HI and hybridized with a *P*-element probe showed that a band with a molecular weight of ~0.5 kb disappeared in the revertants. These results suggested that the modifier mutation might be induced by a *P* insertion.

The 10.5-kb band hybridizing with the *P* element was cloned and the region surrounding the *P* element was sequenced. Homology searching using the GenBank database was performed using the BLASTN program (Altschul *et al.* 1990), and the search showed 100% identity of the cloned region to the *ph* gene. Therefore, the modifier mutation was designated as ph^{P1}.

The structure of the ph^{P1} mutation: Restriction mapping and sequencing of the cloned fragments was performed. The ph^{P1} mutation is induced by the insertion of a defective 1.2-kb *P* element into the untranslated leader sequence of the proximal *ph* gene at 109 bp, according to the cDNA clone map described by Deatrick *et al.* (1991) (Figure 1). The direction of *P*-element transcription coincides with that of *ph*. The 1.2-kb *P* element has exactly the same structure as the *P*-element copy present at the *yellow* locus, *i.e.*, sequences between 829 and 2560 bp are deleted, and the filler sequence TAGCTACAAA is inserted at the breakpoint.

Transcription of the *ph* gene in the ph^{P1} strain: Two major transcripts of 6.4 and 6.1 kb were earlier described, hybridizing with restriction fragments within the 28.6 kb of genomic DNA sequences containing the *ph* genes (Dura *et al.* 1987). The proximal transcription unit encodes two embryonic mRNAs of 6.4 and 6.1 kb, and the distal transcription unit encodes a 6.4-kb embryonic mRNA (Hodgson *et al.* 1997).

The subcloned *Bam*HI-*Sac*I and *Sac*I-*Eco*RV DNA fragments of the proximal *ph* gene were used as probes for Northern blot hybridization (Figure 1A). The *Bam*HI-*Sac*I DNA fragment has no strong homology to the distal *ph* region and includes untranslated leader sequences, a small part of coding region and a part of the first

intron of the proximal *ph* gene (Deatrick *et al.* 1991; De Camillis *et al.* 1992). In the ph^+ strain, the *Bam*HI-*Sac*I DNA fragment hybridizes to one major transcript of 6.4 kb and two minor transcripts of 6.1 and 9.0 kb at all stages of development from embryo to adult (Figure 1B). To understand the nature of the 9.0-kb transcript, we hybridized the Northern blot with the *Sac*I-*Eco*RV DNA fragment that includes part of the *ph* first intron (Figure 1A). The probe hybridized only to the 9.0-kb transcript, confirming that the 9.0-kb transcript is a result of alternative splicing and leaving the first intron nonexcised (Figure 1B). This *ph* transcript has not been described, and its role is unknown.

In the mutant ph^{P1} strain, the *Bam*HI-*Sac*I DNA fragment hybridized to three major bands, 1.0, 6.4, and 10.1 kb (Figure 1B), expressed at the middle pupal stage, *i.e.*, at the time of *yellow* gene expression (Geyer *et al.* 1986). The *Hin*dIII-*Xho*I DNA fragment (Figure 1A) subcloned from the *P* element hybridized to the same three bands, suggesting that all transcripts contained *P*-element sequences and the 5′ nontranslated region of the *ph* gene. The 1.0-kb transcript can be explained by transcription termination within the *P* element, because it did not hybridize to the probe from the 3′ terminus of the *P* element located beyond the signal for polyadenylation (Figure 1A). The 6.4-kb transcript did not hybridize to the DNA fragments from the 3′ terminus of the *P* element and from the first *ph* intron, but hybridized to the *Xho*I DNA fragment (Figure 1A), covering the region from the second to the fifth exon and to the *Bam*HI-*Sac*I DNA fragment (the 5′ nontranslated region of *ph*).

Part of the 6.4-kb transcript, including *P*-element sequences, was cloned by PCR using primers located within the 5′ untranslated region and the second exon of the *ph* gene. Two different PCR products were obtained and subsequently sequenced. The first one contains the 5′ untranslated region of the *ph* gene and the 5′ terminus of the *P* element up to the end of the first exon combined in a correct frame with the second exon of the *ph* gene (Figure 1A). The calculated length of this transcript coincides with that of the wild-type transcript (6.4 kb). The predicted protein from this tran-

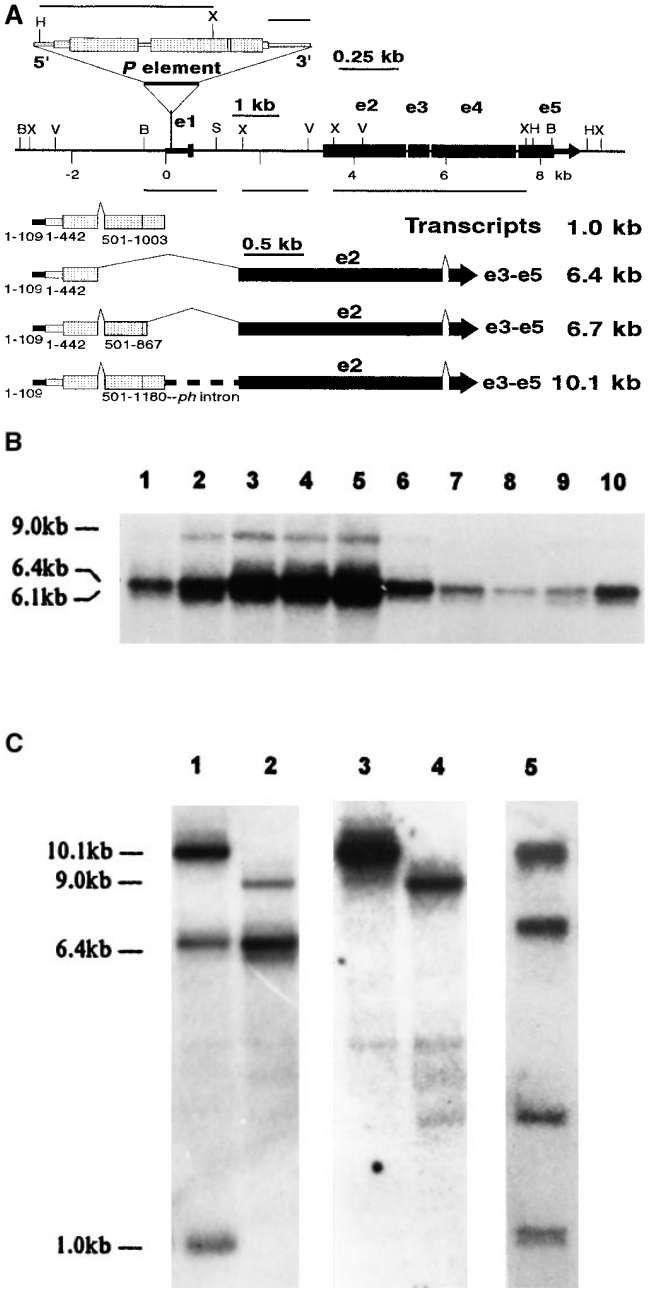

Figure 1.—Schematic representation of the structure and expression of the *ph* gene in the ph^{P1} mutant. (A) The physical map of the proximal *ph* gene and its transcripts in the ph^{P1} mutation. The *ph* coding region is indicated by black boxes, the transcribed untranslated region is indicated by a thick black line, and the intron and nontranscribed regions of *ph* are denoted by a thin line. The *P*-element coding region is shown by white boxes, the 5′ nontranslated part by a thick white line, and other regions of the *P* element by thin white lines. Restriction enzymes are abbreviated as follows: H, *Hin*dIII; S, *Sac*I; X, *Xho*I; B, *Bam*HI; V, *Eco*RV; P, *Sph*I. Below is shown the schematic structure of *ph* transcripts in the ph^{P1} allele. e, exon of *ph* gene; 1.0-kb transcript; 6.4-kb major transcript; 6.7 kb identified by PCR minor transcript; 10.1-kb major transcript. (B) Northern blot analysis of transcripts from the proximal *ph* region in a wild-type *Oregon* strain. Northern blot hybridization of the *Bam*HI-*Sac*I fragment of the *ph* gene with mRNA isolated at different stages of development. 1, 3-day-old females; 2, 3-day-old males; 3, late pupae; 4, middle pupae; 5, early pupae; 6, late third-instar larva; 7, early third-instar larva; 8, second-instar larva; 9, first-instar larva; 10, embryo. (C) Northern blot analysis of transcripts from the proximal *ph* region in ph^{P1} and wild-type *Oregon* strains during middle pupal stage of development. Northern blot hybridization of the *Bam*HI-*Sac*I (1, 2) and *Sac*I-*Eco*RV fragments (3, 4) of the *ph* gene, and *Hin*dIII-*Xho*I fragment of the *P* element (5) with mRNA isolated from ph^{P1} (1, 3, 5) and wild-type *Oregon* (2, 4) strains. The same blot was hybridized with a fragment containing the *ras2* gene (1.6-kb transcript) that is expressed at an approximately constant level during Drosophila development.

script is translated from the ATG codon of the *P* element and contains the DNA-binding region of the *P* element and an almost complete PH protein sequence lacking only 12 amino-terminal amino acids. We designated it as the P-PH protein. The chimeric P-PH protein encoded by the 6.4-kb transcript binds to sequences within the *P* element located at the *yellow* locus and is responsible for the repression of its transcription due to the presence of an almost complete PH sequence.

The second PCR product has the same 5′ terminus, but the first exon of the *P* element is normally spliced to the second exon eliminating the first *P*-element intron. It appears that a new donor splice site is available in the *P* element at position 880 bp of the defective copy, which corresponds to position 2580 bp numbered according to the published complete *P*-element sequence (O'Hare and Rubin 1983). The second exon of the *P* element is fused at this site to the second exon of the *ph* gene with conservation of the correct open reading frame. A protein formed from this transcript is expected to include the DNA-binding domain and two protein-protein-binding regions of the *P*-element transposase (Lee *et al.* 1996) followed by the PH protein sequence lacking the same 12 amino acids as the P-PH protein. The amount of this transcript seems to be low, as the band with the predicted 6.7-kb size was not identified on the Northern blot.

The 10.1-kb transcript hybridized to the probe from the first *ph* intron and to the probe from the 3′ terminus of the *P* element. This transcript is more abundant than the 6.4-kb transcript, in contrast to the 9.0-kb transcript of the ph^{+} strain (Figure 1B). It is possible that the *P*-element insertion interferes with the splicing of the first intron of the *ph* gene. The 10.1-kb transcript contains almost all *P*-element sequences, including the 3′ terminus, part of the first ph exon, the first intron, and all other exons of the *ph* gene. The protein translated from this mRNA is believed to be nonfunctional.

Molecular analysis of ph^{P1} revertants: To check the role of the chimeric P-PH proteins in the repression of *P*-induced *yellow* alleles, we obtained revertants of the ph^{P1} mutation. For this purpose *y z* ph^{P1}; Δ2–3/+ males were crossed to y^{2s14} females. As a result, 36 independent ph^{+P} derivative strains were established. All of them were analyzed by Southern blot hybridization.

Nineteen revertants were found to be caused by an almost complete deletion of the *P* element located at the *ph* locus. Two revertants, ph^{+P11} and ph^{+P27}, cloned by PCR and sequenced, contained 16–18 bp from both *P*-element terminal inverted repeats (Table 2). Four revertants with partial deletion of the *P* element were also cloned by PCR and sequenced. Three of them had the 5′-deletion breakpoint within the 31-bp terminal inverted repeat and the 3′-deletion breakpoint inside the *P*-element body at positions ranging from 209 to 472 bp. In the ph^{+P18} revertant, the deletion was located between 130 and 701 bp (Table 2). Thus, all studied revertants were associated with deletions of sequences from the first exon of the *P* element, which encoded the DNA-binding domain of the transposase. This result confirms the role of the *P*-element DNA-binding domain in the effect of the ph^{P1} mutation. According to results of Southern blot hybridization, the *P*-element sequences were deleted together with the proximal *ph* gene in seven ph^{P1} revertants. Finally, three ph^{P1} revertants were induced by the deletion of a 1- to 3-kb sequence of the 5′ regulatory region, including the promoter of the *ph* gene (data not shown).

Immunolocalization of the P-PH protein in the distal region of the *X* chromosome: If P-PH represses *yellow* by binding, then the P-PH protein should bind to the 1A region, where the *yellow* gene is located. To test this, immunostaining of polytene chromosomes from salivary glands of the y^{2s14} and $y^{2s14}ph^{P1}$ larvae was performed with antibodies to the PH protein (gift of Dr. R. Paro). In agreement with literature data, PH-binding sites were detected in 1A, 2D, 4C, 5A, and 5D (De Camillis *et al.* 1992) of the distal part of the *X* chromosome. The presence of a PH-binding site in the 1A region of the wild-type *X* chromosome did not permit us to ana-

TABLE 2

Analysis of deletion breakpoints in revertants of the ph^{P1} mutation

Allele	5′ break	3′ break	Sequence of breakpoint junction (5′ → 3′) FORWARD
ph^{+12}	16	335 (852)	CATGATGAAATAACAT/gcttatcgactgctgagctgccatcaa/GAGCAT—TTTCATCATG
ph^{+14}	17	472 (715)	CATGATGAAATAACATA/tgtatataacataac/ATGTAA—TTTCATCATG
ph^{+18}	130	701 (386)	CATGATGAAA—CATTAA/CAGCTA—TTTCATCATG
ph^{+22}	19	209 (978)	CATGATGAAATAACATAAG/GCCGGA—TTCATCATG
ph^{+11}	16	2892 (16)	CATGATGAAATAACAT/ATGTTATTTCATCATG
ph^{+27}	18	2892 (16)	CATGATGAAATAACATAA/ATGTTATTTCATCATG

P-element orientation in the ph^{P1} mutation coincides with that of the *ph* gene. The deletion breakpoints are numbered according to the published complete *P*-element sequence (O'Hare and Rubin 1983). Numbers in the 5′- and 3′-break columns indicate, respectively, the 5′- and 3′-nucleotide positions of the internal deletion breakpoints. The number in parentheses after the 3′ breakpoint position indicates the number of base pairs of the *P*-element sequences, and the lower case letters denote filler sequences present at the breakpoint in a number of alleles. —, sequences between the breakpoint and the end of the *P* element.

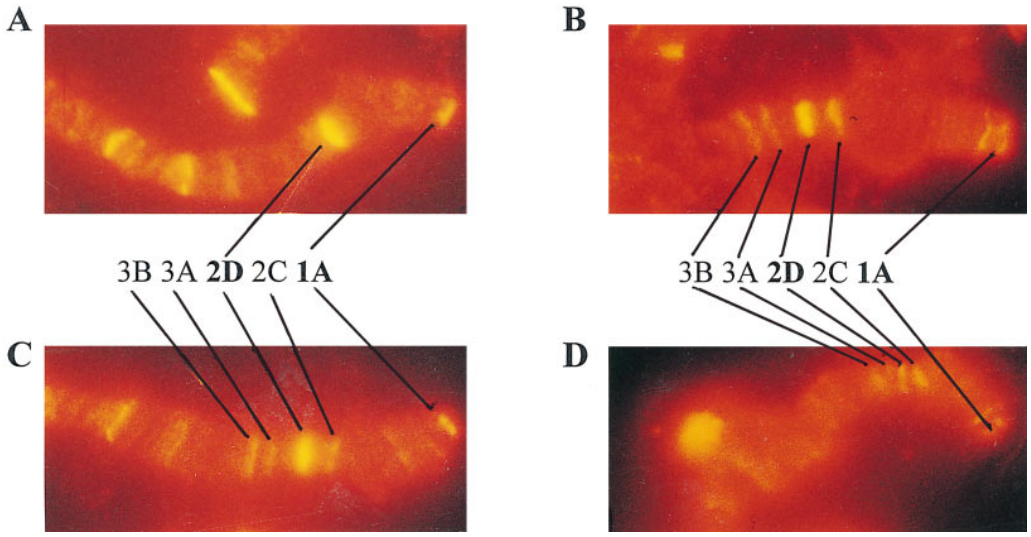

Figure 2.—Immunohistochemical localization of PH, PSC, and PC proteins on the distal region of the *X* chromosome in wild-type (A) and ph^{P1} (B, C, and D) strains. In the wild-type strain, PH binding was detected in 1A and 2D regions. The PC and PSC proteins were detected in the same 1A and 2D regions. The additional PSC- (B), PH- (C), and PC- (D) binding sites appearing in the ph^{P1} strain were detected in the same regions: 2C, 3A, and 3B.

lyze the possibility of formation of a new site in the *yellow* locus.

However, three new sites for PH protein in the 2C, 3A, and 3B regions of the distal part of the *X* chromosome were detected in the $y^{2s14}ph^{P1}$ strain when compared to the y^{2s14} one (Figure 2). *In situ* hybridization experiments with the y^{2s14} and $y^{2s14}ph^{P1}$ strains showed that the same regions had *P*-element copies. Thus, *P*-element-containing regions have acquired the ability to bind the PH protein in the ph^{P1} mutant chromosome, confirming that the chimeric protein is able to interact with *P*-element sequences.

Effect of the ph^{P1} mutation on different *yellow* alleles: The next step of our work was to combine the ph^{P1} mutation with different *P*-element-containing *y* alleles. The majority of *y* alleles used for this purpose originated from strains with super unstable *P*-induced mutations in the *yellow* locus raised in the y^2 background (Georgiev *et al.* 1992, 1997). The effect of ph^{P1} on *yellow* gene expression was different and depended on the molecular structure of the *y* alleles.

We first analyzed whether the orientation of the *P* element in the *yellow* locus contributed to the observed level of P-PH-mediated inactivation of *yellow* expression. The *P* element in the y^{2sA3} allele has the same structure as in the y^{2s14} or the y^{2s20} mutations, but its direction of transcription is inverted and coincides with the direction of *yellow* transcription (Figure 3). The ph^{P1} mutation enhanced the mutant phenotype of the y^{2s14}, y^{2s20}, and y^{2sA3} alleles to the same extent: the thoracic and leg bristles became yellow (Table 1). Thus, *P*-element orientation did not influence the effect of the ph^{P1} mutation.

The y^{76d28} allele is of special interest because the *P* element has inserted in the 5′ transcribed nontranslated portion of the *yellow* gene (Geyer *et al.* 1988). In y^{76d28} flies, pigmentation of all adult cuticular structures is tan (Table 1). The ph^{P1} mutation further enhanced the mutant phenotype of y^{76d28} flies to approximately the null level, suggesting that the *P*-element position relative to the *yellow* promoter was not critical for the repression effect of the $ph^{P1.}$

Two previously described y^{2s14} revertants, y^{+s7} and y^{+s8} (T. Belenkaya and P. Georgiev, unpublished data), were induced by the excision of *gypsy* with one of the long terminal repeats (LTRs) remaining at the insertion site (Figure 3). These alleles were used to test the possibility of an almost normally transcribed gene to be affected by the ph^{P1} mutation. The effect of the ph^{P1} mutation on the bristle pigmentation was less pronounced and the pigmentation of body and wings was only slightly decreased (Table 1).

Some of the *y* alleles in our collection have two or even three tandemly inserted *P*-element copies (Georgiev *et al.* 1997) that allowed us to study the role of *P*-element copy number in the ability of ph^{P1} mutation to repress *yellow* expression. Two *y* alleles, y^{2s7} and y^{2s11}, possessed a pair of 1.2-kb *P* elements in the inverted head-to-head orientation (Figure 3). The *P*-element duplication on its own did not influence the y phenotype (Table 1), but the combination of y^{2s11} or y^{2s7} with ph^{P1} completely inactivated *yellow* expression: the flies became unpigmented. Another previously obtained allele, y^{ds62}, was cloned and shown to be induced by the insertion of three 1.2-kb *P* elements (Figure 3). Although y^{ds62} flies have darker pigmentation than y^2 flies, the ph^{P1} mutation also completely repressed *yellow* expression (Table 1). These findings indicate that the presence of two or three *P* elements strongly enhances the repressive effect of the ph^{P1} mutation on *yellow* expression.

Pairing-dependent repressive effect of the ph^{P1} mutation: To determine whether the repressive effect of the ph^{P1} mutation depends on allelic pairing, we studied the effect of the ph^{P1} mutation on phenotypes of females that have different combinations of *y* alleles.

In the presence of the ph^{P1} mutation, females homozygous for *y* alleles with one *P*-element copy (y^{2s14}, y^{+s7})

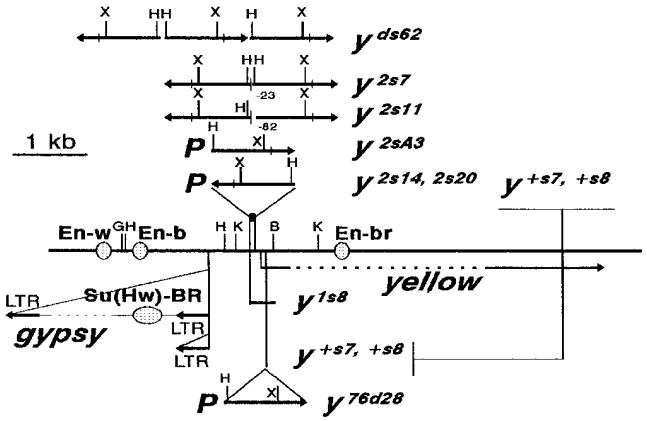

Figure 3.—Structure of *y* alleles. Exons of the *yellow* gene and direction of transcription are indicated by arrows. Transcriptional enhancers and the su(Hw)-binding region of *gypsy* are marked by ovoid structures. En-w, enhancer for the wing blade; En-b, enhancer for the body cuticle; En-br, enhancer for bristles. Insertions responsible for different alleles are represented by arrows indicating their direction. The *gypsy* element is inserted in the 5′ region of the *yellow* gene in the y^2 allele and its derivatives. Arrows flanking *gypsy* are LTRs. Deleted DNA sequences are shown by dotted lines. Restriction enzymes are abbreviated as follows: H, *Hin*dIII; G, *Bgl*II; X, *Xho*I; K, *Kpn*I; B, *Bam*HI.

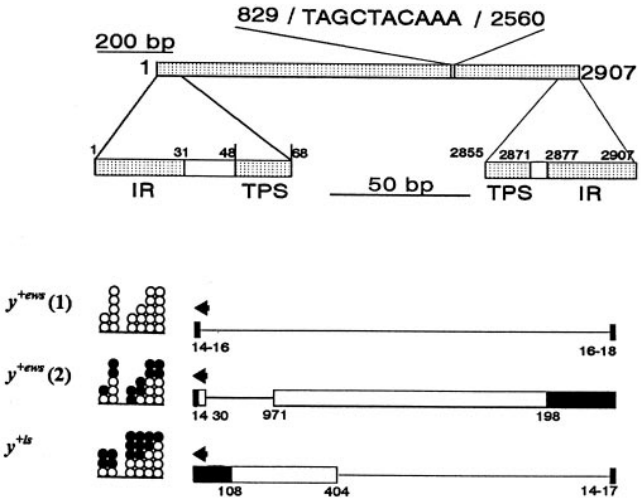

Figure 4.—Schematic presentation of the ph^{P1} effect on deletion derivatives of the *P* element in *y* alleles. Above is a structure of the 1.2-kb *P* element. The double line in the center of this box indicates the internal deletion breakpoint of this *P* element. IR, 31-bp inverted repeat; TPS, transposase-binding site. Below are the *P*-element sequences present in the alleles indicated at the left. The empty boxes represent maximal *P*-element sequences remaining in the deletion derivatives; the black boxes represent the minimal sequences. The figures below the boxes are the numbers of base pairs remaining in the *P*-element sequences from the corresponding end to the breakpoint. The thin lines between the boxes represent deleted *P*-element sequences. The arrows show the direction of the *P* element in the corresponding alleles. The number of black and white circles indicates the level of pigmentation in 1, the body; 2, wings; 3, thoracic bristles; 4, leg bristles; 5, wing bristles; 6, abdominal bristles. The number of black circles shows the inhibitory effect of the ph^{P1} mutation in y^*/y^{2s11} females. One circle represents one point as in Table 1.

show stronger mutant phenotypes than heterozygous females having the same *y* alleles in combination with y^1, which is induced by a point mutation in the *yellow* coding region (Lindsley and Zimm 1992). In combination with the y^{1s8} mutation, which contains a *P*-element insertion and, simultaneously, a deletion of the *yellow* gene from −69 to +118 bp, the repressive effect of ph^{P1} on *yellow* is enhanced. It appears that single *P*-element copies induce a weak cooperative repression of *yellow* transcription in the presence of ph^{P1}.

The y^{2s11} mutation is caused by a double *P*-element insertion (Figure 3). y^{2s11}/y^{2s14} females in the presence of ph^{P1} had a phenotype similar to that of y^1 flies (Table 3). We studied heterozygotes containing y^{2s11} with the described above y^{+s7} allele carrying one *P*-element copy. *yellow* gene expression in y^{2s11}/y^{+s7} females with the ph^{P1} mutation was also significantly reduced (Table 3). When the y^{ds62} allele (three copies of the *P* element) is used instead of y^{2s11} in combination with the y^{+s7} allele, further enhancement of the ph^{P1} effect on *yellow* expression occurs (Table 3). Thus, the level of the pairing-dependent repression of *yellow* transcription directly correlates with the number of *P*-element copies.

The ph^{P1} mutation fails to affect the pigmentation of the y^2/y^{2s11} or y^2/y^{ds62} heterozygous females, indicating that the presence of *P*-element sequences in both homologues is necessary. To test the minimal *P*-element sequences required for transinhibition, we used derivatives of the y^{+s7} and y^{2s14} alleles resulting from *P*-element excisions (T. Belenkaya and P. Georgiev, unpublished data). *P*-element excisions in the y^{+s7} strain have led to the appearance of y^{+ews} derivative alleles. The y^{+ews} flies are characterized by a weak reduction of the body cuticle and bristle pigmentation compared to the parental y^{+s7} flies (Table 3). Molecular analysis of the y^{+ews} alleles has shown that they can be divided into two classes (Figure 4). Two y^{+ews} alleles of the first class resulted from an almost complete deletion of the *P* element, only 14–18 bp being retained at each terminus. The ph^{P1} mutation did not influence the pigmentation of flies homozygous for y^{+ews} alleles or their combination with either y^{2s11} or y^{2s62} alleles (Figure 4; Table 3). Thus, the terminal 18 bp of *P*-element sequences are not sufficient for the repression of *yellow* transcription by the ph^{P1}.

Four y^{+ews} alleles of the second class have a partial *P*-element deletion with one breakpoint in the 5′ inverted repeat, while the 3′ terminus retains from 198 to 971 bp (Figure 4). The ph^{P1} mutation also failed to influence the pigmentation of males or homozygous females with any of these y^{+ews} mutations. This result suggests that at least 971 bp from the 3′ part of the *P* element are not enough to mediate repression of *yellow* transcription by the P-PH protein. In contrast, the presence of the ph^{P1} mutation strongly reduced the

TABLE 3

ph^{P1}-mediated transrepression of transcription in females heterozygous for *y* alleles

Females with *y* and ph^{P1} mutation	Pigmentation					
	Body	Wings	Th	L	W	Ab
y^{2s14}/y^{2s14}	1	1	1	2	4	4
y^{2s14}/y^{1}	1	1	1	2	5	5
y^{2s14}/y^{1s8}	1	1	1	1	3	4
y^{2s14}/y^{2s11}	0	0	0	0	0	0
y^{+s7}/y^{+s7}	5	5	3	4	5	5
y^{+s7}/y^{1}	5	5	5	5	5	5
y^{+s7}/y^{1s8}	4	5	3	3	5	5
y^{+s7}/y^{2s11}	3	4	1	2	4	4
y^{+s7}/y^{ds62}	3	3	0	0	2	2
y^{2}/y^{2s11} or y^{ds62}	1	1	5	5	5	5
$y^{+ews}(1)/y^{+ews}(1)$	3	5	2	3	5	5
$y^{+ews}(1)/y^{1}$	2	5	2	3	5	5
$y^{+ews}(1)/y^{2s11}$ or y^{ds62}	2	5	2	3	5	5
$y^{+ews}(2)/y^{+ews}(2)$	3	5	2	3	5	5
$y^{+ews}(2)/y^{1}$	2	5	2	3	5	5
$y^{+ews}(2)/y^{2s11}$	1	3	0	1	4	4
$y^{+ews}(2)/y^{ds62}$	0	2	0	0	2	2
y^{+ls}/y^{+ls}	3	3	5	5	5	5
y^{+ls}/y^{2s14}	1	1	4	4	5	5
y^{+ls}/y^{2s11}	1	1	2	2	4	4
y^{+ls}/y^{ds62}	0	0	0	0	3	2

The abbreviations and numbers indicating the levels of pigmentation are as described in Table 1. $y^{+ews}(1)$ and $y^{+ews}(2)$ denote y^{+ews} alleles of the first and second class described in the text.

pigmentation of flies with heterozygous combinations of y^{+ews} and either y^{2s11} or y^{ds62} alleles. Thus, 198 bp of the *P*-element sequence adjacent to the 3′ terminus, including the transposase-binding region, are sufficient for the ph^{P1}-mediated pairing-dependent inhibition of *yellow* expression.

Three derivatives of the y^{2s14} allele (Figure 4) designated as y^{+ls} (the pigmentation of body cuticle and wings is intermediate between y^{1} and y^{+}) have internal deletions, with one breakpoint within the 31-bp terminal inverted repeat at the 3′ end and the 5′ breakpoint inside the *P*-element body at positions 108–404 bp. The ph^{P1} mutation failed to affect the pigmentation of the y^{+ls} males and females, suggesting that both *P*-element termini are important for repression (Table 3).

The y^{+ls} alleles, in combination with y^{2s11} (two *P*-element copies) and especially with y^{ds62} (three *P*-element copies), are strongly affected by ph^{P1} (Table 3). Thus, it can be concluded that 108 bp from the 5′ end of the *P* element are enough for transrepression of *yellow* transcription by the ph^{P1} mutation.

The ph^{P1} mutation induces the formation of PC-G protein complex on *P*-element sequences: As it has been shown above, when the *P*-element insertion is present in the *yellow* locus, the P-PH protein bound to *P*-element sequences induces inhibition of *yellow* transcription. This raises the question of whether other PC-G proteins are involved in this process.

In order to answer this question, immunostaining of polytene chromosomes from salivary glands of y^{2s14} and y^{2s14} ph^{P1} larvae was performed with antibodies to the PC and PSC proteins (gift of Dr. R. Paro). In both strains, y^{2s14} and $y^{2s14}ph^{P1}$, PC and PSC sites were detected in the same regions as for P-PH: 1A, 2D, 4C, 5A, and 5D (Zink and Paro 1989; De Camillis *et al.* 1992; Rastelli *et al.* 1993). In the $y^{2s14}ph^{P1}$ strain, the same additional sites were identified as for P-PH protein: 2C, 3A, and 3B, which coincide with the regions of *P*-element localization (Figure 2). Thus, in the ph^{P1} mutant, the *P*-element-containing regions acquire the ability to bind not only the P-PH protein but also other PC-G proteins, in particular PC and PSC, obviously through their interaction with P-PH. This conclusion is supported by the fact that at least in the case of the y^{2s14} and y^{+s7} alleles induced by a single *P*-element insertion, the repressive effect of ph^{P1} on *yellow* transcription can be diminished by mutations in the *Pc* (Pc^{1} and Pc^{3}) and the *Psc* (Psc^{1}) genes (Table 4). In contrast, null mutations (Breen and Duncan 1986; Wu *et al.* 1989; Bornemann *et al.* 1996) in some other *Pc*-G genes, such as *Scm* (Scm^{XF24} and Scm^{D1}) and *E(z)* [$E(z)^{S1}$ and $E(z)^{1}$], have no distinct effect on the ph^{P1}-mediated repression of *yellow* transcription. None of the mutations influenced the inhibitory effect of the ph^{P1} mutation on the *y* alleles derived by a double or triple *P*-element insertion (data not shown).

DISCUSSION

The experiments described here demonstrate that the chimeric P-PH protein consisting of the *P*-element transposase-binding domain and a major portion of the PH protein acts as a transcriptional silencer and establishes *Pc*-G-dependent repression of a *yellow* gene containing a *P*-element insertion.

Locke *et al.* (1988) proposed that the PC-G proteins might form a multimeric complex. Support for this idea has come from observations that PC and PH bind to identical sites on polytene chromosomes and coimmunoprecipitate (Franke *et al.* 1992). The PSC and SU(Z) binding sites on polytene chromosomes also substantially overlap with those of PC and PH (Martin and Adler 1993; Rastelli *et al.* 1993). The recruiting mechanism for assembling a *Pc*-G complex in Drosophila has been shown by the use of a PC protein fused to the GAL4 DNA-binding domain (Muller 1995) or of the chimeric HP1-PC protein, which binds both heterochromatic and euchromatic sites (Platero *et al.* 1996). Immunoprecipitation experiments using *in vivo* cross-linked chromatin indicate that PC, PSC, and PH proteins are associated with identical regulatory elements of the selector gene *engrailed* in tissue culture cells (Strutt

TABLE 4

Suppression of ph^{P1} effect by mutations in *Pc*-G

		Pigmentation			
		y^{2s14}		y^{+s7}	
Mutant	Type of mutation	Th	L	Th	L
+		1	2	1	3
$E(z)^{1}$	Gain of function[a]	1	2	1–3	4
$E(z)^{S1}$	Antimorphic[a]	1	2	1–3	4
Pc^{1}		1	2	3–4	5
Pc^{3}		1–3	3	4–5	5
$Su(z)2^{1}$	Gain of function[b]	1–5	2–5	4–5	5
$Su(z)2^{1.a1}$		2–3	4	5	5
$Su(z)2^{4}$		2–4	4–5	3–5	4–5
$Su(z)2^{5}$	Gain of function[a]	1	2	3–5	4–5
Psc^{1}	Complex[c]	—	—	3–5	4
Scm^{XF24}	Null[d]	1–2	2	2–4	4
Scm^{D1}	Null[e]	1–2	2	2–4	4
Su(z)302	Gain-of-function allele of *Scm*[a]	3–4	4–5	5	5

The abbreviations and numbers indicating the levels of pigmentation are as described in Table 1.
[a] Wu *et al.* 1989.
[b] Brunk *et al.* 1991.
[c] Adler *et al.* 1989.
[d] Bornemann *et al.* 1996.
[e] Breen and Duncan 1986.

and Paro 1997). Recently it was found that PSC protein contacts PH and PC through specific conserved domains (Kuba and Brock 1998).

Analysis of the amino acid sequence showed that the PH protein contained glutamine repeats, a single putative zinc finger, and the SPM domain in the carboxy terminus (De Camillis *et al.* 1992). However, the PH protein on its own does not exhibit a sequence-specific DNA-binding activity *in vitro* (Franke *et al.* 1992; Rastelli *et al.* 1993). In contrast, the chimeric P-PH protein has acquired the ability to bind specifically to the DNA independently of the other PC-G proteins. The 1.2-kb *P*-element inserted in the ph^{P1} allele has a deletion between 829 and 2560 and resembles the previously described KP element, which lacks amino acids within interval 800–2560 bp (Black *et al.* 1987). The truncated transposase produced by the KP element contains the intact DNA-binding domain and two regions providing protein-protein interactions that are important for effective binding of truncated transposase to *P*-element sequences (Lee *et al.* 1996). The major 6.4-kb mRNA of the ph^{P1} gene expected to encode the chimeric P-PH protein contains only 119 N-terminal amino acids of the *P* transposase that include the DNA-binding domain and a part of a putative leucine zipper dimerization domain (Andrews and Gloor 1995). It is possible that the protein-protein interaction region, which is important for dimerization and high-affinity DNA binding, is supplied by the SPM domain of the PH protein. The minor mRNA identified by PCR encodes a chimeric protein that has 199 amino acids of the transposase amino terminus, including the DNA-binding region and both protein-protein interaction regions. Thus, this P-PH protein can bind the *P*-element termini with high affinity using only the *P*-element domains.

The P-PH protein bound to *P*-element sequences is able to recruit at least two other PC-G proteins as suggested by the coincident sites of immunolocalization of the P-PH, PC, and PSC proteins with the *P*-element insertion sites on polytene chromosomes. The effect of mutations in the *Pc*-G genes on the ph^{P1}-mediated inhibition of *yellow* transcription does not exclude the possibility that other PC-G proteins also participate in organization of this complex.

Our genetic observations suggest that both *P*-element termini enclosing the transposase-binding sites are necessary for the repression of *yellow* transcription by ph^{P1}. The degree of repression directly correlates with the number of *P*-element copies at the *yellow* locus. These data suggest a cooperative mechanism for ph^{P1}-mediated assembly of repressive complexes on *P*-element sequences. Thus, the *P*-element sequences function as known PREs in the ph^{P1} backround.

Transposons containing PRE sites often show an enhancement of silencing when the fly is homozygous for a transposon insertion, indicating that the homologously paired PREs interact to produce a more stable and more repressive *Pc*-G complex (Fauvarque and Dura 1993; Chan *et al.* 1994; Kassis 1994; Pirrotta 1997; Sigrist and Pirrotta 1997). We have also found a transinterac-

tion between *y* alleles exhibiting weak and strong ph^{P1}-mediated repression. However, a *y* allele in the homologous chromosome requires at least some of the transposase-binding sequences of the *P*-element termini for this effect to take place. These regions can play the role of "weak PRE" that can induce repression in the presence of another "strong PRE" in the homologue.

In this article we describe a novel mechanism for the action of a mobile element insertion on the control of gene expression. This is the formation of chimeric genes encoding the functional domains of both a mobile element protein and a protein encoded by a target gene. The insertion of a truncated *P* element, like our 1.2-kb element or KP, into leader sequences or introns of regulatory genes may generate chimeric transcriptional proteins with an altered DNA-binding activity. Such proteins are able to bind to any *P*-element copy present in the genome and also to other sequences with homology to those recognized by the *P*-element transposase. As a consequence, new regulatory regions may appear in this way. We are now carrying out selective screens to obtain such mutations for other regulatory genes.

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