polyhomeotic regulatory sequences induce developmental regulator-dependent variegation and targeted *P*-element insertions in *Drosophila*

Marie-Odile Fauvarque and Jean-Maurice Dura

Centre National de la Recherche Scientifique (CNRS) URA 1134, Laboratoire d'Embryologie Moléculaire. Université Paris XI-Bât. 445, 91405 Orsay Cedex, France

Variegation of the *miniwhite* gene is observed in a euchromatic context in transformant lines that contain a *P* transposon including regulatory sequences of the *polyhomeotic* (*ph*) gene upstream of the resident *miniwhite* gene (*P[ph]*). This variegated phenotype is not affected by most of the genetic modifiers of heterochromatic position-effect variegation (PEV) nor by removal of the Y chromosome. Interestingly, it is sensitive to *ph* and *Polycomb* (*Pc*) mutations, which are known to affect homeotic gene regulation. Regulatory DNA of *ph* can also mediate transvection of the *miniwhite* gene. This transvection is abolished in a *ph* but not in a *zeste* mutant background. In addition, *P[ph]* inserts preferentially in sites corresponding to PH/PC protein-binding sites as defined at the polytene chromosome level. These insertions induce an unusually high proportion of mutations in genes affecting homeotic gene regulation. In particular, one insertion is located within the *tramtrack* locus, which is thought to regulate *fushi tarazu*, an *Ultrabithorax* activator. We suggest that a multimeric complex containing PH and PC proteins, at a minimum, causes a local and clonally inherited heterochromatinization, which maintains the repressed state of transcription of the homeotic genes.

[**Key Words:** Homeotic gene regulation; local heterochromatinization; *Polycomb* group genes; target gene identification; transvection]

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In *Drosophila*, the segmental identity of the body depends on the spatially restricted expression of the homeotic genes of two complexes: *Antennapedia* (*ANT-C*) and *bithorax* (*BX-C*) (Lewis 1978; Kaufman et al. 1980; Sanchez-Herrero et al. 1985; for reviews, see Akam 1987; Duncan 1987; McGinnis and Krumlauf 1992). The emergence of the specific expression pattern of the homeotic genes in the blastoderm requires the activity of early-acting segmentation genes (Duncan 1986; Ingham and Martinez-Arias 1986; Irish et al. 1989). The maintenance of this specific pattern depends on two groups of trans-regulator genes: the *trithorax* group (*trx-G*) and the *Polycomb* group (*Pc-G*) (Lewis 1978; Struhl 1981; Duncan 1982; Jürgens 1985; Kennison and Tamkun 1988; for reviews, see Ingham 1985; Paro 1990; Kennison and Tamkun 1992), which maintain the active and inactive state, respectively, of homeotic gene transcription during the rest of development (Struhl and Akam 1985; Wedeen et al. 1986; Dura and Ingham 1988; Huang and Dawid 1990; Jones and Gelbart 1990; Mazo et al. 1990; McKeon and Brock 1991; Simon et al. 1992; Tamkun et al. 1992). The *polyhomeotic* (*ph*) locus has been defined as a member of the *Pc-G* because it exhibits the posteriorly homeotic transformations characteristic of *Pc-G*

mutant phenotypes (Dura et al. 1985, 1987, 1988). Members of the *Pc-G* act synergistically on homeotic gene transcription—double or triple mutant combinations showing more dramatic homeotic transformations than the simple addition of each mutant effect (Jürgens 1985). Mutations in the *trx-G* antagonize and suppress mutations in the *Pc-G* (Ingham 1983), and most members of the *trx-G* were screened according to this criterion (Kennison and Tamkun 1988).

The two proteins PH and PC were found to be associated with ~100 common discrete sites on the polytene chromosomes. The PH/PC target sites include the two homeotic complexes but also several loci corresponding to *trx-G* and *Pc-G* genes (Zink and Paro 1989; Zink et al. 1991; DeCamillis et al. 1992). In particular, the locus 2D, where *ph* is located, is included in PH/PC target sites, indicating that *ph* expression may be regulated by PH and PC proteins. PH and PC proteins were shown to bind chromatin in a DNA-specific manner, although neither PH nor PC was shown to bind directly to naked DNA (Zink et al. 1991; DeCamillis et al. 1992). As they coprecipitate with ~10–15 other proteins, suggesting that they act in a multimeric complex, it is likely that at least one of the other components binds to the DNA (Franke

et al. 1992]. The discovery of a homologous domain, the "chromodomain," between PC and a nonhistone heterochromatin-associated protein HP1 (Paro and Hogness 1991) has provided significant advances in the understanding of the molecular mechanism by which members of the *Pc-G* may act on their targets. Removal of this chromodomain from PC abolishes its attachment to the chromatin (Messmer et al. 1992). *Pc-G* proteins may act by inducing a local heterochromatin-like structure ensuring the transcriptional repression of their target gene, particularly the homeotic genes, which would thus be maintained in a clonally repressed state (Paro 1990). However, no genetic or molecular evidence that members of the *Pc-G* are effectively able to induce a local heterochromatinization has yet been provided.

In this paper we describe variegation of the *miniwhite* gene in a euchromatic context in lines that contain a *P* transposon, including regulatory sequences of *ph* upstream of the *miniwhite* gene (*P[ph]*). Normally, variegated eyes are observed when a chromosomal rearrangement places the euchromatic *white* gene next to heterochromatin, and this phenomenon is called position-effect variegation (PEV) (for reviews, see Eissenberg 1989; Henikoff 1990; Tartof and Bremer 1990; Reuter and Spierer 1992). In the case of PEV, the clonal *white* expression results from the spreading of the heterochromatic domain to include, in some cells, the *white* gene, thereby inhibiting its expression. Once established, the heterochromatin is then maintained as such in the daughter cells leading to a clonal expression of *white* in the eye. Here, we show that the variegated phenotype of the *P[ph]* lines is not the result of PEV because the insertions are located in euchromatic areas; moreover, this variegation is neither modified by most genetic modifiers of PEV nor by removal of the Y chromosome, which greatly enhances PEV. Interestingly, the variegated phenotype of the *P[ph]* transgenic lines depends on a normal dosage of PH and PC proteins. This suggests that *Pc-G* genes may act by inducing a local heterochromatinization. We also found that *ph* regulatory DNA can mediate transvection of the *white* gene. This transvection is abolished in *ph* but not in a *zeste* mutant background.

The genetic and molecular identification of all the components of the *Pc-G* and *trx-G* is crucial for the understanding of their mode of action. As a number of these loci correspond to PH/PC target sites, the identification of PH/PC target genes may provide a means to find other members of the *Pc-G* and *trx-G*, putative new homeotic genes, and potential new classes of target genes. Here, we describe the utilization of the *P[ph]* transposable element as a putative powerful method for the screening of PH/PC target genes. This element inserts preferentially into sites that correspond to PH/PC-binding sites as detected at the polytene chromosome level. Some of them are within genes that act in homeotic gene regulation. Thus, the *P[ph]* transposable element may appear to be useful for both the identification of PH/PC target loci and the understanding of the molecular mechanism by which homeotic genes are regulated.

Results

ph regulatory DNA induces variegation of the *white* gene in transgenic lines

In an effort to study the regulation of *ph* expression throughout development, we made transgenic constructs containing different regulatory regions of the *ph* locus driving the *lacZ* gene (*P[ph]*; Fig. 1). In one set of experiments (418 and 419 constructs), the transforming vector contained *ph* regulatory DNA upstream of the *miniwhite* gene driven by its own promoter. In a high proportion of *P[418]* transgenic lines (50/130 = 38%), issuing from either transformation or transposition, we observed variegation of *white* expression. One of six 419 transformed lines also showed this phenotype. Two types of variegated phenotype can be distinguished (Fig. 2): the patterned phenotype, in which all of the individuals exhibit a reproducible patterned *white* expression, and the mottled phenotype, in which expression of *white* appears randomly, very likely in a clonally inherited manner. For most strains, a mixture of the two phenotypes is observed. Variegation of the *white* gene following transformation or transposition is normally a rare event (Hazelrigg et al. 1984; Levis et al. 1985). No more than 1% of anomalous expression of *white* was detected when hundreds of lines carrying a *P-lacW* insertion were made (Dura et al. 1993). We injected the pCaSpeR-AUG- β -gal vector (Thummel et al. 1988) without any *ph* sequences, and we obtained 14 independent lines. None of them showed any sign of *white* gene variegation. Therefore, our working hypothesis is that *ph* regulatory sequences are directly responsible for the variegated expression of the *miniwhite* gene.

The variegated phenotype induced by ph regulatory sequences is not attributable to PEV

Variegated expression of a euchromatic gene is seen when a chromosomal rearrangement places it next to heterochromatin (for recent reviews, see Eissenberg 1989; Tartof et al. 1989; Henikoff 1990; Reuter and Spierer 1992). This PEV is dependent on genetic elements like the Y chromosome and suppressor and enhancer loci. We investigated whether or not the *ph*-induced variegation was similar to PEV. First, for the inserts inducing variegated eyes that we mapped ($n = 24$), all but one were in euchromatic, rather than in heterochromatic, areas of the polytene chromosomes (for details, see Table 2, below). Second, for seven strains that we have tested (*I7.1*, *T2.2;31*, *T3*, *T13.4*, *T30*, *T42.1*, *T72.2*), the absence of the Y chromosome has no effect on XO male eye color. In contrast, as expected, enhancement of PEV (in *w^{m4}* males) attributable to absence of Y was observed. Third, we tested the effect of 22 suppressors and 9 enhancers of variegation (listed in Materials and methods) on the *T3* line, and 4 suppressors and 6 enhancers on three other 418 lines (*I7.1*, *T53.3*, and *T30*). These four 418 lines exhibit variegation visible in heterozygous flies. In almost all cases, no effect of these modifiers of PEV was detectable in 418 lines. However,

Figure 1. (A) Schematic representation of *ph* proximal unit and of fragments 418, 419, 800, and 804 containing various *ph* sequences. Distances are indicated in kb. Solid boxes represent regions homologous with the distal *ph* unit. Restriction sites are abbreviated as follows: (X) *Xba*I; (P) *Pst*I; (E) *Eco*RI; (K) *Kpn*I. (B) Position of 418 and 419 fragments in transposon *P[ph]*. Arrows indicate the direction of transcription.

compared with the effect on PEV (in w^{vco} males), a lower effect is seen in the cases of *Su-var(2)1001* and *E-166/7* which, respectively, partially suppress and enhance *T3*-variegated phenotype. Interestingly, the effect is not always the same as for the effect on PEV: *Su-var(2)201* weakly enhances the variegation of *T3*; *E-90* suppresses the variegation of *T3*; and *E(3)1⁰¹* suppresses the variegation of *T3* and *T30*. Furthermore, modifications are visible only for some of the 418 lines tested. It seems clear that the modifiers of PEV that we have tested (31 in total) do not act on 418 line variegation in the same way as they do on PEV.

Environmental treatment can also modify variegating phenotypes. Temperature is the classical case, with higher temperatures typically suppressing and lower temperatures enhancing the variegating phenotype (Tartof and Bremer 1990). Seven strains showing variegated eyes (the same as described above) were grown at 17°C, 25°C, and 28°C. In contrast to PEV, in all strains, the variegation is strongly enhanced when the tempera-

ture increases (Fig. 3). A control strain bearing a *miniwhite* transgene did not show any eye color variation at the different temperatures (data not shown).

From all of these results, we can conclude that *ph* regulatory DNA induces variegation that is not similar to that induced by PEV.

ph regulatory DNA-induced variegation is modified by developmental regulators

PC and PH proteins are part of a multimeric complex that binds chromatin at ~100 sites in the polytene chromosomes (Franke et al. 1992). One of these sites corresponds to the *ph* locus itself (Zink and Paro 1989; DeCamillis et al. 1992). Therefore, we tested the effect of *ph* and *Pc* mutations on *ph*-induced variegated eye phenotype. All the variegated strains tested, except one, showed modification of the eye color in *ph* and/or *Pc* mutant backgrounds (Table 1). When the change occurs, it is always darker, that is, variegation is suppressed in a

Figure 2. 418 variegated phenotype at 25°C. (A) *I7.1/Y*, patterned expression in which all individuals exhibit a reproducible white expression; (B) *T15.2/T15.2*, mottled expression in which expression of white appears randomly, probably in a clonally inherited manner; (C) *T3/T3*, mixture of patterned and mottled white expression.

Figure 3. DREV is enhanced while PEV is suppressed at higher temperatures. (A) w^{vco} , 17°C; (B) w^{vco} , 25°C; (C) T30, 17°C; (D) T30, 25°C.

$ph^{-}/+$ or ph^{-}/Y context and it is lighter, that is, variegation is enhanced in a $Pc^{-}/+$ context. As for the 418 lines, the variegating 419 line (Table 1) is sensitive to both ph and Pc mutations. Interestingly, in three 419 nonvariegating lines (of a total of five), a variegated phenotype is induced in a Pc^{-} background.

In contrast to ph and Pc mutations, no effect was detected in fifteen 418 variegated lines in a trx^3 mutant background (lines tested: T3, T13.5, T26, T30, T33.1;24j, T40, T42.1, T43, T44, T53.3, T63, T66.1, T69.3, T72.1, T72.2).

Transgenic lines for a $P[w^{+}]$ element containing the regulatory region of *engrailed* ($P[en]$) also show patterned eyes in a high proportion of cases (Kassis et al. 1991). The product of *en* is ectopically expressed in a ph mutant background (Dura and Ingham 1988), and the *en* locus (48A) corresponds to a PH/PC protein-binding site. This suggests that PH could also modify the variegated phenotype induced by *en* regulatory sequences. We observed that the eye color becomes darker in ph^{410} males in three $P[en]$ lines, G6, F3, and SR1-c, but not in a fourth, F4 (Kassis et al. 1991). However, the suppression of the variegated phenotype by ph is less marked than in the case of $P[ph]$ transgenic lines. These results indicate that the variegation observed in the case of both $P[ph]$ and $P[en]$ transgenic lines is probably induced by some common developmental factors, particularly the ph product. There is no visible effect of Pc^K mutation on these four $P[en]$ lines.

Because ph regulatory DNA-induced variegation is not attributable to PEV but is modified by developmental regulators like ph and Pc , we propose calling this new kind of variegation developmental regulator effect variegation (DREV).

lacZ gene expression also variegates

None of the four series of transgenic lines, 418, 419, 800, and 804 (see Fig. 1), contains sufficient ph regulatory sequences to initiate a wild-type ph pattern of *lacZ* expression in embryos as it is described for ph transcripts (Deatrick 1993). However, in six variegating lines tested (I2.2;31, T3, T26, T30, T40, T66.1), we observed variegated expression of the *lacZ* gene in imaginal discs (Fig. 4), which suggests that ph regulatory sequences induce variegation to both the left and right.

ph regulatory DNA induces miniwhite gene transvection

The *miniwhite* gene is not fully functional, and the resulting eye color is dependent on gene dosage: homozygous flies ordinarily have darker eyes than heterozygous flies (Pirrota 1988). For most of the variegated 418 lines (31/44), homozygous individuals have lighter eye color than do heterozygous flies (Fig. 5, cf. T3/+ and T3/T3). This phenomenon is very likely to be similar to the transvection effect induced by *en* regulatory DNA on

Table 1. Variegated phenotype and effect of *ph* and *Pc* mutation on various 418 and 419 lines at 25°C

	Insert	Hemizygous	Heterozygous	Homozygous	<i>ph</i> ⁴¹⁰	<i>Pc</i> ^K		
418	<i>I7.1</i>	pat	pat	pat	L	D	L	
	<i>T2.2;31</i>	—	—	mot	L	=	L	
	<i>T13.4</i>	mot	—	mot	L	D	L	
	<i>T26</i>	—	—	mot	L	D	L	
	<i>T40</i>	pat/mot	—	pat/mot	L	D	L	
	<i>T43</i>	—	—	pat/mot	L	D	L	
	<i>T63</i>	pat	pat	pat	L	D	=	
	<i>T72.1</i>	—	—	pat/mot	L	D	L	
	<i>I2.1.2</i>		pat	pat	D	D	=	
	<i>I33.1</i>		pat	pat	L	=	L	
	<i>T3</i>		pat/mot	pat/mot	L	D	L	
	<i>T13.5</i>		pat/mot	pat/mot	L	D	L	
	<i>T15.2</i>		—	mot	L	D	=	
	<i>T30</i>		mot	mot	L	D	L	
	<i>T33.1;2</i>		—	mot	L	=	L	
	<i>T33.1;5</i>		—	mot	L	=	=	
	<i>T33.1;24j</i>		pat/mot	lethal		D	L	
	<i>T42.1</i>		pat/mot	pat/mot	D	D	L	
	<i>T44</i>		pat	lethal		D	=	
	<i>T53.3</i>		pat	pat	L	D	=	
	<i>T66.1</i>		pat/mot	pat/mot	L	D	=	
	<i>T69.3</i>		—	pat	L	D	=	
	<i>T72.2</i>		mot	mot	L	D	=	
	419	<i>419/6.1</i>		pat	—	D	D	L
		<i>419/5.1</i>		—	—	D	=	V
		<i>419/8.1</i>		—	—	D	=	V
		<i>419/19.1</i>		—	—	D	=	V

Hemizygous mutants are described in the cases of X-linked insertion. Homozygous mutants have lighter (L) or darker (D) eyes than heterozygous. Heterozygote insert lines have darker (D) eyes in *ph*/+ or *ph*/Y background and lighter (L) in *Pc*/+ background, or they are identical (=). Variegation appears in three of five originally nonvariegating 419 lines in *Pc*/+ background (V); (pat) Patterned white expression; (mot) mottled white expression.

white expression (Kassis et al. 1991). No transvection effect was detected in the 14 *P*[pCaSpeR-AUG-β-gal] control lines. Transheterozygotes for two transfecting *P*[*ph*] inserts located at the same site on the polytene chromosome, also show transvection of the *miniwhite* gene. This was demonstrated for the two X-linked lines,

I7.1 and T63, mapping at 12D, and for the three lines T15.2, T53.3, and T69.3 (all combinations tested) mapping at 35C. The eye pattern of the lines that have the same insertion site are almost identical. Thus, the patterned aspect of the eye probably depends on the genomic sequences surrounding the transposon.

We tested the effect of *ph*⁴¹⁰ on the transvection of 10 transfecting autosomal 418 lines (I33.1, T3, T13.5, T15.2, T30, T42.1, T53.3, T66.1, T69.3, T72.2). When a *ph*⁴¹⁰ mutant background, either in homozygous or hemizygous condition, is introduced in such transfecting lines, the transvection effect is completely abolished: In *ph*⁴¹⁰ context, individuals homozygous for the insert have darker eyes, actually wild type, than the heterozygous flies (Fig. 5, cf. *ph*⁴¹⁰/Y;T3/+ and *ph*⁴¹⁰/Y;T3/T3). This suggests that PH protein plays an active role in causing white expression to be repressed in 418 homozygous lines, probably via *ph* regulatory DNA sequences. Transvection of the white gene was also described for some of the *P*[*en*] insertions described above. At least for one line, F3, transvection is abolished in *ph* mutant background. In contrast to the *ph* mutation, null mutations of either *Pc* (*Pc*^K) or *trx* [*Df*(3R)*red-P93*] genes have no effect on *ph*-mediated transvection as tested on five X-linked transfecting lines (T13.4, T26, T40, T43, T72.1).

Figure 4. Variegated expression of the *lacZ* gene in two wing imaginal discs from the T40 line.

Figure 5. The transvection effect mediated by *ph* regulatory sequences is abolished in *ph* mutant background. *T3/+* (A) have darker eyes than *T3/T3* (C), but *ph*⁴¹⁰/*Y*; *T3/+* (B) males have lighter eyes than *ph*⁴¹⁰/*Y*; *T3/T3* (D) males. Note that variegation is completely suppressed in the *ph*⁴¹⁰ background.

The *zeste* gene has been implicated in mediating transvection for many different genes (Gans 1953; Gelbart and Wu 1982; Wu and Goldberg 1989; Pirrota 1990). The effects of *z*^a, an amorphic allele of *zeste*, was tested on seven X-linked 418 lines (*I7.1*, *T2.2;31*, *T13.4*, *T26*, *T40*, *T43*, *T72.1*). Only the transvection of the *T2.2;31* line is suppressed in a *z*^a background. No effect of *z*^a on the eye color of the other six strains was detected. These data suggest that the Z protein is not an essential component for the interaction mediated by the *ph* sequence.

A number of P[ph] elements insert preferentially within genes involved in homeotic gene regulation

Because chromosomal localization was of primary importance to correctly interpret the observed DREV phenomenon, we localized 19 independent variegating insertions on polytene chromosomes (Table 2). Surprisingly >50% (11) are localized in the same polytene chromosomal section as a PH/PC protein-binding site (Zink and Paro 1989; DeCamillis et al. 1992), which suggests that *P[ph]* inserts nonrandomly and preferentially into PH/PC target genes. Another proof of the directed nature of the insertion events is that two locations (12D and 35C) had two and three independent inserts, respectively.

However, cytogenetic localization is rather imprecise at the molecular level because two apparently coincident

cytological binding sites might be separated by as much as 200 kb. To examine the hypothesis of *P[ph]* preferential insertion in PH/PC targets, we looked at the nature of *P[ph]*-induced mutations. Because a high number of PH/PC chromosomal binding sites are located where *Pc-G* and *trx-G* loci map (Zink and Paro 1989; DeCamillis et al. 1992), the *Pc-G* and *trx-G* genes are probably part of a genetically regulated network. Consequently, a certain number of the *P[ph]* inserts may be within *Pc-G* and *trx-G* gene, mutants which enhance and suppress *ph* and *Pc* phenotypes, respectively. We tested all of the 418 lines for which insertion had produced a lethal or visible phenotype (16 lines) and lethal excisions of two X-linked lines (*I7.1* and *T2.2;31*). We have also tested, in homozygous condition, 10 randomly chosen, variegating viable 418 lines. Each of these lines was tested for enhancement or suppression of the homeotic transformations induced by *ph* and/or *Pc* mutations [for details, see Materials and methods]. Among 130 *P[ph]* lines, we analyzed 28 lines and found 3 *Pc-G* members and 1 *trx-G* member (the results are summarized in Table 3). This proportion is at least 100 times higher than that which can be obtained with a similar *P* element not carrying *ph* regulatory sequences (for more details, see Discussion). We note that two of these four lines do not variegate, which suggests that preferential insertion can occur independently of the variegated phenotype. Therefore it seems clear that an unusually high proportion of genes acting in

Table 2. Cytogenetic localization of variegating insertions on polytene chromosomes

Insertions	Chromosome	In situ localization	PH/PC protein-binding sites ^a
T43	1	5D	5D
T40	1	9D	
I7.1	1	12D	12D
T63	1	12D	12D
T26	1	12E	
T2.2;31	1	14B	14B
T13.4	1	20D	
T44	2	24D	
T42.1	2	28B	
T53.3	2	35C	35C
T15.1	2	35C	35C
T69.3	2	35C	35C
T41.2 ^b	2	35CD	35C
T13.5	2	36BC	36AC
I33.1	2	38F	38F
I2.1.2	2	56F	56F
T33;24j	2	60B	
T15.2	3	65CD	65D
T3	3	65F	
T66.1	3	84DE	84D
T33.1;2	3	84F	84F
T30	3	86CD	86C
T33.1;5	3	88C	
T11.1 ^b	3	88D	
804-41.5 ^b	3	100D	100D

^aPH/PC protein-binding sites are defined as in Zink and Paro (1989) and DeCamillis et al. (1992).

^bNonvariegating lines mapped for their particular phenotype.

homeotic gene regulation are mutated by a *P[ph]* transposon.

An 804 insertion locates in the tramtrack gene

Transgenic lines 800 and 804 (Fig. 1A) do not show any sign of variegation, probably because modified transposons *P[800]* and *P[804]* contain the HSP70 promoter upstream of the *white* gene (Kuhn et al. 1988). This does not exclude the possibility that preferential insertion may be obtained with these two transposons. Therefore, we mapped one of the nineteen 800 and 804 insertions on polytene chromosomes because it is embryonic lethal. Interestingly this insertion 804-41.5 is located at 100D in a PH/PC protein-binding site. Moreover, we demonstrated by Southern analysis that it maps within the *tramtrack* (*ttk*) locus. In two different genomic digestions probed with the p69 form of *ttk* cDNA (Read and Manley 1992), a new band appears in the 804-41.5 line as compared with the two controls: the strain *w¹¹¹⁸* in which transformation was carried out, and the strain 804-29.1, another 804 transgenic line (Fig. 6). The gene *ttk* was isolated as a gene encoding a *fushi tarazu* (*ftz*) promoter-binding protein (Harrison and Travers 1990;

Table 3. Effect of four *P[ph]* insertions on homeotic transformations induced by *ph* and/or *Pc* mutations

<i>P[ph]</i> insertion	MT to Pro leg ^a	MS to Pro leg ^b	Antenna to leg ^c
2.2;31/+ control	—	—	1.53**
T11.1/+ control	2.67*	2.82**	1.15
T41.2/+ control	2.00	1.55	1.13
T66.1/T66.1 control	1.50**	1.00**	1.09*
	2.47	1.50	1.27
	2.83**	—	—
	1.71	—	—

Transformations were scored as described in Materials and methods. Each number represents the mean class of 50 legs or antennae. The probability (*P*) that the two populations containing or not containing the insertion are identical was calculated with the Chi² test. (MS) Mesothoracic; (Pro) prothoracic; (MT) metathoracic. (*) *P* < 0.05; (**) *P* < 0.001.

^aIn *ph⁴¹⁰/Y* context.

^bIn *Pc^K/+* context.

^cIn *ph⁴⁰¹/+; Pc³/+* context.

Brown et al. 1991). It also codes for a related protein that binds to the promoter of another pair-rule gene: the *even-skipped* (*eve*) gene (Read and Manley 1992). This 804-41.5 insertion, which has probably occurred via a preferential insertion, provides the first mutant allele for *ttk* as no mutant has been described previously. The embryonic lethal allele that we obtained exhibits strong cuticular defects (Fig. 7): Heads do not develop properly; thoracic and abdominal segments seem to be present, but ventral denticle bands are significantly reduced; spiracles and filzkörpers are still detectable; and dorsal closure is not always achieved. Thus, *ttk* product appears to be an essential factor for the development of the embryo.

Figure 6. Insertion 804-41.5 is located in the *ttk* gene. Genomic DNAs of *w¹¹¹⁸* (A), 804-41.5/+ (B), and 804-29.1/+ (C) were digested by *Eco*RI or *Bam*HI and probed with the cDNA p69 of *ttk*. Arrows indicate the appearance of a new band.

Figure 7. Cuticular phenotype of *ttk* embryos. Ventral is at the bottom and anterior is at the left. The head fails to develop properly. Ventral denticle bands are quasi-absent.

Discussion

PH and *PC* proteins modify the variegated phenotype mediated by *ph* regulatory sequences

ph and *Pc* are members of the *Pc-G* genes that maintain the repressed state of the homeotic genes during embryogenesis of *Drosophila*. The *PC* protein has a region—the chromodomain—which shows 65% similarity to the nonhistone heterochromatin-associated protein HP1 (Paro and Hogness 1991). The HP1 protein is encoded by *Su(var)205*, a suppressor of PEV (Eissenberg et al. 1992). Before this homology was known, Locke et al. (1988) proposed a model, based solely on genetic experiments, suggesting a mechanistic link between the *Pc-G* gene family and the modifiers of PEV. This chromodomain is responsible for the binding of *PC* protein to chromatin (Messmer et al. 1992). On the basis of this homology, a model has been proposed where the repressed state of homeotic genes is maintained and clonally inherited by means of a local induction of a heterochromatin-like structure (Paro 1990). This local DNA compaction could be induced by a multimeric complex composed of different proteins of the *Pc-G*, including at least *PH* and *PC* proteins (Franke et al. 1992). It was never demonstrated, however, that *Pc* or other members of the *Pc-G* are ef-

fectively able to induce a local heterochromatinization. Genomic insertion of a modified *P* element carrying *ph* regulatory sequences upstream of the *miniwhite* gene (*P[ph]*) induces variegated eyes in 38% of the transgenic lines. This particular variegated phenotype is not the result of insertion into heterochromatin and is generally not modified by genetic modifiers of PEV. In contrast, mutations in either *ph* or *Pc* genes, respectively, suppress and enhance this variegated phenotype. We propose, therefore, to call this new variegation, DREV. We have also observed that *ph* mutation partially suppresses variegation of *P[en]* transgenic lines (Kassis et al. 1991), suggesting that this variegation is the result of events similar to those in the variegation induced by *ph* sequences.

To explain the random expression of the *miniwhite* gene, we hypothesized that certain proteins are able to induce a local heterochromatinization at the level of the transposon. The variegation phenotype normally occurs in the near vicinity of heterochromatin; thus, by analogy, DNA compaction at the level of the transposon may result in a structure similar to that of heterochromatin. Therefore, we will now use the term of local heterochromatinization to describe this phenomenon. Do *PH* and *PC* proteins take part in this local heterochromatinization? A 50% reduction of these proteins is able to modify and even induce, in the case of *PC*, a variegated phenotype, and both have been shown to bind the chromatin of their target loci in a DNA sequence-dependent manner (Zink et al. 1991; DeCamillis et al. 1992). As *PH* and *PC* are known to bind in 2D, where the *ph* locus lies (Zink and Paro 1989; DeCamillis et al. 1992), we suggest that the presence of *ph* regulatory sequences allows the binding of *PH* and *PC* proteins and, perhaps, other members of the *Pc-G*. This binding may generate a local heterochromatinization leading to the observed variegated phenotype (Fig. 8). Although we cannot exclude that the effect of *PH* and *PC* proteins is not direct, it is clear that this variegated *miniwhite* gene expression depends on *ph* and *Pc* product. This supports very strongly the idea that these two proteins act by inducing a local heterochromatinization. DREV should provide a new and useful tool to study the mechanism by which homeotic genes are regulated through local heterochromatinization.

Figure 8 Hypothetical model for creating a local heterochromatinization leading to the variegating phenotype. Binding of a *PH/PC* multimeric complex on *ph* regulatory sequences would generate a local heterochromatinization, including the *white* gene, as well as the *lacZ* gene, and therefore lead to the observed variegated phenotype. Open boxes indicate compacted and, therefore, inactive chromatin.

DREV and PEV may be the result of analogous chromatin constitution

DREV is not only influenced by developmental regulators like PH and PC proteins; for example, increasing the temperature induces an enhancement of DREV while inducing a suppression of PEV (Tartof and Bremer 1990). This may indicate that the chromatin organization that induces DREV is formed by multimeric units, the concentration of which depends on a mass-action model (Locke et al. 1988).

DREV is also influenced by some modifiers of PEV (5 of 31 tested), but the effects of these modifiers are not always the same as for PEV (3 of 5). These results suggest that some proteins implicated in PEV may also be implicated in the DREV phenomenon. For example, in the case of DREV, once the heterochromatinization signal is given by developmental regulators, the effective DNA compaction is likely to depend on certain structural proteins implicated in the physical conformation of the heterochromatin. If some of these proteins are identical to those implicated in the formation of centromeric heterochromatin, that would indicate that the local heterochromatin made in DREV has, effectively, a physical constitution similar to the heterochromatin. However, the fact that DREV is influenced in a different way by temperature and by some modifiers of PEV, and not at all by removing the Y chromosome, provides evidence that physical components operating in DREV and PEV contain some structural differences.

P[ph] transposon may insert preferentially into PH/PC target genes

Approximately 100 common target loci have been defined on polytene chromosomes for the two proteins PH and PC. Neither PH nor PC has been shown to bind DNA directly, but both proteins coprecipitate in the same multimeric complex that is thought to mediate the observed DNA binding (Franke et al. 1992). Known loci are members of PH/PC targets, especially the two homeotic gene complexes *BX-C* and *ANT-C*, and many *Pc-G* and *trx-G* genes (Zink and Paro 1989; DeCamillis et al. 1992). As assayed by chromosomal in situ hybridization experiments, the insertion of the *P[ph]* transposon at the chromosomal level frequently occurs at PH/PC protein-binding sites. Thus, it was possible that it occurred in *Pc*- or *trx-G* genes. We found three enhancers and one suppressor of *ph* and/or *Pc* mutations. These 4 loci were isolated from 28 transgenic lines chosen non-randomly from a pool of 130 418 lines. This exceptionally high rate of mutation induced by the insertion of the *P[ph]* transposon, in genes implicated in homeotic gene regulation, is to be compared with other results that we obtained: First, among 50 lines carrying one *P[w⁺]* lethal insertion, none was found to interact with *ph⁴¹⁰*. Second, we screened 10,000 chromosomes carrying at least one *P[w⁺]* insertion and found four suppressor genes, and no enhancers of *ph⁴¹⁰* (M.-O. Fauvarque and J.-M. Dura, unpubl.).

This illustrates particularly well the advantage of using the *P[ph]* element for finding genes that interact with *ph*. Thus, preferential insertions may provide a powerful method for identifying PH/PC target genes. In support of this idea, we obtained a *ttk* mutant allele, the first described to our knowledge, indicating that *ttk* is likely to be a PH/PC target gene. In addition, the presence of the transposon greatly facilitates further genetic analysis and molecular cloning.

Preferential insertion in the *en* region (Hama et al. 1990) and in genes exhibiting a stripe expression pattern (Kassis et al. 1992) was observed with transposons carrying *en* regulatory sequences [*P[en]*]. We suggest that regulatory proteins bring together transposons carrying *ph* or *en* regulatory sequences and the endogenous target gene sequences into which preferential insertion occurs. In this model the regulatory proteins common to the transposon and the target gene must be present in the germ line of flies in which transposition occurs. This is the case, at least in the female germ line, for both *ph* and *Pc* protein products (Paro and Zink 1992; M. DeCamillis and H. Brock, pers. comm.). PH and PC proteins regulate *ph* expression as well as *en* expression (Busturia and Morata 1988; Dura and Ingham 1988; Moazed and O'Farrell 1992; this study). Therefore, PH and PC are good candidates for mediating the preferential insertion of the two transposons. This would explain the overlapping of preferential insertion sites for the two transposons *P[ph]* and *P[en]*, illustrated by the high number of insertions in 35C (3/130 for *P[ph]* and 2/49 for *P[en]*). However, not only PH and PC may mediate preferential insertion but any other protein of the specific multimeric complex that is supposed to bind *ph* (or *en*) regulatory sequences. All of the preferential insertions of the two transposons should not, then, be identical. Moreover, the *T11.1* insertion that enhances *ph* phenotype is not located at a known PH/PC target site. We suggest that protein(s) other than PH/PC may mediate preferential insertion into a member of the *Pc-G*. Another possible explanation is that some target genes of PH/PC are not detectable, or only weakly, on salivary gland polytene chromosomes.

Regulation of the ph gene by the PH/PC multimeric complex

Contained among the 100 PH/PC-binding sites is the *ph* locus (Zink and Paro 1989; DeCamillis et al. 1992). However, it has not been demonstrated that PH and PC effectively regulate the expression of *ph*. On the contrary, no effect of *Pc* mutations on *ph* expression in embryos is observed (Franke et al. 1992). However, our results suggest that PH and PC are regulators of *ph* later in development, as they are able to modify *miniwhite* gene expression when close to *ph* regulatory sequences. Furthermore, PH and PC are good candidates for mediating preferential insertion into PH/PC target genes by binding to *ph* sequences.

PH and PC have opposite effects on *miniwhite* gene expression, and by extension they may have opposite effects on *ph* expression. *Pc* may be an activator of *ph*,

whereas *ph* may be a repressor of its own expression. However, if *ph* is regulated by *Pc*, mutation of *Pc* will provoke a change in the amount of the *ph* product. Thus, the effect observed in *Pc*⁻ context not only reflects the diminution of the *Pc* product but also a change in the amount of *ph* product (and other *Pc*-regulated genes). Because our results strongly suggest that *ph* regulates itself, conclusions about the mechanism of *ph* regulation by *Pc* are not immediately straightforward. It would not be surprising, however, that *Pc* acts positively on *ph* transcription because *Pc* and *ph* act synergistically on homeotic gene regulation (Dura et al. 1985). If *Pc* were to repress *ph* transcription, then *Pc* mutations would have a suppressor effect on homeotic transformation induced by the *ph* mutation. In this scheme, *Pc* (and *ph*) should act positively on the transcription on those *Pc*-G genes in which mutations act synergistically with *Pc* (and *ph*) in homeotic gene regulation.

PH acts on the ph regulatory DNA-mediated transvection effect

Homologous chromosomes are intimately paired in somatic cells of insects. The transvection effect originally defined by Lewis (1954) (for a recent review, see Wu and Golberg 1989) suggests a functional role for somatic pairing. In most of the variegating 418 lines, homozygous individuals have lighter eye color than heterozygous ones. This phenomenon can be classified as a transvection effect because according to the current definition, it is an "allelic interaction that shows dependence on chromosomal pairing or allele proximity" (Judd 1988). Although the phenomenon that we observe is similar to that mediated by the *zeste* gene at the *white* locus, the *Z* protein was not found to be an essential component of this interaction. Here, we showed that PH protein acts on the transvection effect induced by *ph* regulatory sequences. In a *ph* mutant background, the transvection effect is completely abolished while *ph* mutation does not affect the transvection of *white* induced by the *z*¹ mutation (data not shown). We note that a transvection effect of the same kind mediated by a fragment of *en* regulatory DNA has been described recently (Kassis et al. 1991). At least in one case, the same suppression of transvection was seen in a *ph* mutant background. We suggest that the normal function of this transvection is similar and may be mediated by the same kind of proteins in both cases. This is the first description of a transvecting role for the PH protein. This function does not seem to be shared by the PC protein because no effect of *Pc* mutation on *ph*-mediated transvection was observed. Thus, PH and PC proteins may act in a different way within the PH/PC multimeric complex. We propose that PH protein has a tendency to aggregate. This can be the case for other components of the multimeric complex as well. This aggregation will result in more complex forms when two copies of the transgene are in proximity to one another and, therefore, will strengthen repression. Thus, the transvection effect may represent a mechanism mediating the regulatory effect of PH.

Local heterochromatinization, preferential insertions, and transvection may all be explained by a tendency of the proteins that compose the multimeric complex to aggregate. The name "aggregulate" has recently been proposed for such regulating complex (Jacob 1993). This provides a simple explanation of how the repression of homeotic genes is maintained during successive cell divisions: As soon as the newly formed DNA strand is synthesized, a complete multimeric complex will form under the influence of that part that remains on the old strand. This will ensure a clonal maintenance of the repressed state of determination.

Materials and methods

Fly strains and culture

All strains were maintained on standard culture medium at 17°C, 25°C, or 28°C. All variants used are described in Lindsley and Zimm (1992), except for the amorphic *Pc*^K allele, which is described in Zink and Paro (1989).

Vector and constructs

418 and 419 fragments were cloned in the *P*-element vector pCaSpeR-AUG-β-gal (Thummel et al. 1988) in the orientation shown in Figure 1. 800 and 804 fragments were cloned in the *P* element vector pW-ATG-lac2 (Kuhn et al. 1988). Positions of restriction sites used for constructs are given in parentheses as referred to in Deatrick et al. (1991): 418, 2.9-kb fragment *Xba*I(3467)-*Pst*I(6272); 419, 4.0-kb fragment *Eco*RI(1397)-*Kpn*I(5329); 800, 4.9-kb fragment *Eco*RI(1367)-*Pst*I(6272); and 804, 8.8-kb fragment, which results from a partial digestion with *Pst*I, *Eco*RI(1397)-*Pst*I(10177 or 10309).

Generation of transgenic lines

P-element-mediated transformation was carried out essentially as described by Rubin and Spradling (1982). *w*¹¹¹⁸ embryos were injected with 150 mg/ml of helper plasmid [pUCHsPΔ2-3 (D. Rio, pers. comm.)] and 300 mg/ml of construct. The construct 418 was injected into a *ry*⁵⁰⁶*Sb* *P*[*ry*⁺ Δ2-3(99B)/*TM6,Ubx*] stock containing a stable source of transposase (Robertson et al. 1988). Transformants were identified on the basis of their eye color and established as homozygous or as balanced heterozygous stocks. New transgenic lines were generated by mobilization of *P*-element construct 418, using the stable source of transposase Δ2-3.

Strains that show interacting effects with *ph* and/or *Pc* mutants were outcrossed for eight generations with a *w*¹¹¹⁸ strain and were retested.

Modifiers of PEV tested with 418 lines

Twenty-two suppressors and nine enhancers of PEV were kindly provided by G. Reuter and J. Gausz (pers. comm.). Suppressors were *Su-var(2)101*, *Su-var(2)505*, *Su-var(2)1301*, *Su-var(2)b601*, *Su-var(2)401*, *Su-var(2)501*, *Su-var(2)502*, *Su-var(2)503*, *Su-var(2)b301*, *Su-var(2)b4801*, *Su-var(2)b204*, *Su-var(2)b701*, *Su-var(2)b801*, *Su-var(2)201*, *Su-var(2)1001*, *Su-var(3)316*, *Su-var(3)103*, *Su-var(3)c1101*, *Su-var(3)902*, *Su-var(3)1002*, *Su-var(3)401*, and *Su-var(3)111*. Enhancers were *E-915*, *E-183/31*, *E-56/9*, *E-90*, *E-129/1*, *E-166/7*, *E-102/1*, *E-104*, and *E-(3)1⁰¹*. For all of the enhancers and for four suppressors [*Su-var(2)101*, *Su-var(2)b204*, *Su-var(3)103*, *Su-var(3)1002*], we determined that

Fauvarque and Dura

they produced the expected effect on males carrying the w^{vco} allele. Only the *Su-var(2)101* locus did not suppress the variegated phenotype of the w^{vco} allele. The effect on eye color is observed in males, issued from the cross between males carrying a modifier of PEV and females carrying the *418* insertion.

Analysis of β -gal expression

Embryos (0–12 hr) were stained with antibodies against β -galactosidase as described by Ingham and Martinez-Arias (1986). Staining of imaginal discs was performed essentially as described in Ashburner (1989).

Interaction with ph and Pc: modification of homeotic phenotypes

The partial transformation of second and third legs to first legs in ph^{410}/Y or in $Pc^K/+$ males results in the appearance of sex comb teeth on the second and third legs of males where they are not normally found. These transformations are scored as follows: (1) In the case of ph^{410} males, third legs are ranked into three classes depending on the number of extra teeth. First class, 0–2 teeth; second class, 3–5 teeth; third class, more than 5 teeth. (2) In the case of $Pc^K/+$ males, second legs are ranked as for transformation 1. The partial transformation of antennae to legs in females $ph^{401}/w^{1118};Pc^3/+$ results in deformation of existing segments and the appearance of leg segments on the antennae. This transformation (3) is scored as follows: transformed antennae are ranked into two classes. First class, deformation more or less marked with no more than one new leg structure; second class, appearance of more than one new leg segment.

Preparation of chromosome spreads and in situ localization of transposon on polytene chromosomes

Drosophila polytene chromosome spreads were prepared from salivary glands of third-instar larvae, which were grown at 17°C. Salivary glands were dissected in Ringer's solution (7.5 grams of NaCl; 0.35 grams of KCl; 0.21 grams of $CaCl_2$ in 1 liter of sterile H_2O). For fixation, three pairs of glands were transferred into a droplet of 45% acetic acid on a coverslip (20 mm²) for 5 min. The coverslip was picked up with a poly-L-lysine-coated slide. [Coating of slides: Clean slides were dipped into a 0.1% poly-L-lysine solution (Sigma) diluted 5× in H_2O ; and air dried.] The coverslip was moved slightly with a pencil, and tapped extensively for at least 1 min with the eraser of a pencil without moving the coverslip. The slide was turned over onto blotting paper, and thumb pressure was applied to the preparation. The coverslip was flipped off with a razor blade after freezing in liquid nitrogen. Slides were immersed immediately into ethanol/acetic acid (3 : 1) for at least 10 min, dehydrated in 100% ethanol for 10 min, and air-dried. Slides can be kept for 2 months before pretreatment. Chromosomes were heat treated in 2× SSC at 68°C for 30 min. Slides were then dipped in 2× SSC at room temperature for 2 min. Chromosomes were acetylated for 8 min in freshly made 0.1 M triethanolamine (pH 8.0)/0.125% and acetic anhydride (vol/vol). Slides were washed for 4× for 4 min each in 2× SSC at room temperature and dehydrated 2× for 5 min each in 70% ethanol and 5 min in 95% ethanol, and air-dried. Chromosomes were denatured in freshly prepared 0.07 N NaOH at room temperature for 3 min. Slides were washed 3× for 5 min each in 2× SSC at room temperature and dehydrated and air-dried as described above. The slides must be used for hybridization within 24 hr after pretreatment. Construction 418 was used as probe. Labeling was performed

with the Random priming kit from Boehringer, using 1 μ l of BiodUTP as labeled nucleotide (1 mM biotin-16-dUTP, Boehringer), and 500 ng of linearized DNA in a total volume of 20 μ l. The reaction was stopped with 25 mM EDTA (final concentration). DNA was precipitated with 2 volumes of ethanol in the presence of tRNA (2 mg/ml) and NaOAc (0.3 M) for 2 hr at –20°C. After centrifugation, the pellet was washed with 70% ethanol and resuspended in 150 μ l of buffer Hbio [600 mM NaCl, 1× Denhardt's, 50 mM $NaPO_4^-$ (pH 7.2), and 5 mM $MgCl_2$ conserved at –20°C]. The probe can be conserved several months at –20°C. Twenty microliters of the probe, which was denatured and vortexed, was applied per slide. For all incubation steps, slides were kept in a humid atmosphere with a coverslip placed over the chromosomes. Hybridization was performed for 12–16 hr at 58°C with coverslips sealed with rubber cement. After hybridization, coverslips were removed and slides were washed for 3× for 20 min each in 2× SSC at 53°C. Slides were placed at room temperature for 1× for 2 min each in PBS (prepared from a 10× stock: 1.3 M NaCl; 0.07 M Na_2HPO_4 ; 0.03 M NaH_2PO_4), 1× for 2 min each in PBS/0.1% Triton-X-100 and 1× for 5 min each in PBS. Slides were air-dried and 100 μ l of 1 : 200 diluted Detec-I-Hrp (Kit Enzo EBP 820-1), in dilution buffer, was applied per slide. Slides were incubated for 30 min at room temperature in a moist chamber. Coverslips were removed and slides were washed at room temperature for 5 min in 2× SSC, for 5 min in PBS/0.1% Triton-X 100 and for 5 min in PBS. Slides were air-dried and 100 μ l of the substrate mixture was applied per slide and the drop was covered with a coverslip. [The substrate mixture was prepared as follows: 3 μ l of 30% H_2O_2 was added to 1 ml of a solution of diaminobenzidine tetrahydrochloride (DAB) 0.5 grams/liter in PBS just before utilization.] After 8 min of reaction, coverslips were removed and slides were washed in dH_2O for 2× for 3 min each. (Caution: DAB is a powerful carcinogen. Work in the hood and use bleach to inactivate the DAB.) Chromosomes were stained for 30 sec in a solution of Giemsa (Merck 1 : 20 dilution in 10 mM sodium phosphate buffer at pH 6.8) and washed immediately for 30 sec in distilled water and air-dried. If necessary, staining time can be longer. Preparations were mounted in Euparal.

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Note added in proof

Since this paper was accepted for publication, two mutations (*ttk¹* and *ttk^{1ell}* of the *ttk* gene have been described (W.-C. Xiong and C. Montell 1993. *Genes & Dev.* 7: 1085–1096). The *P*-element mutant we describe here will therefore be referred to as *ttk⁸⁰⁴*.

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Fauvarque and Dura

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M O Fauvarque and J M Dura

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