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DEVELOPMENTAL BIOLOGY

Developmental Biology 261 (2003) 426–442

www.elsevier.com/locate/ydbio

Identification and characterization of *polyhomeotic* PREs and TREs

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Received for publication 4 October 2002, revised 8 May 2003, accepted 12 May 2003

Abstract

The *polyhomeotic (ph)* gene is a member of the *Polycomb* group of genes (*Pc-G*), which are required for the maintenance of the spatial expression pattern of homeotic genes. In contrast to homeotic genes, *ph* is ubiquitously expressed and it is quantitatively regulated. *ph* is negatively regulated by the *Pc-G* genes, except *Psc*, and positively regulated by the antagonist *trithorax* group of genes (*trx-G*), suggesting that *Pc-G* and *trx-G* response elements (PREs and TREs) exist at the *ph* locus. In this study, we have functionally characterized PREs and TREs at the *ph* locus that function in transgenic constructs. We have identified a strong PRE and TRE in the *ph* proximal unit as well as a weak one in the *ph* distal unit. The PRE/TRE of both *ph* units appear atypical compared with the well-defined homeotic maintenance elements because the minimal *ph* proximal response element activity requires at least 2 kb of sequence and does not work at long range. We have used chromatin immunoprecipitation experiments on cultured cells and embryos to show that *Pc-G* proteins are located in restricted regions, close to the *ph* promoters that overlap functionally defined PRE/TREs. Our data suggest that *ph* PRE/TREs are *cis*-acting DNA elements that modulate rather than silence *Pc-G*- and *trx-G*-mediated regulation, enlarging the role of these two groups of genes in transcriptional regulation.

Keywords: *Drosophila*; *Polycomb*; *trithorax*; *polyhomeotic*; PREs; TREs; Quantitative regulation

Introduction

The *Polycomb* group (*Pc-G*) and the *trithorax* group (*trx-G*) genes encode two groups of transcriptional regulatory proteins which are thought to act by inducing a higher order chromatin structure which maintains gene expression states during cell division. *Pc-G* genes were initially characterized based on their homeotic mutant phenotypes, which are due to the misexpression of homeotic genes. *Pc-G* proteins (PC-G) may maintain an inactive transcriptional state of homeotic genes by inducing a locally condensed chromatin structure, while *trx-G* proteins (TRX-G) maintain an active transcriptional state by opening up chromatin (for review, see Brock and van Lohuizen, 2001). Recent studies have demonstrated that several proteins that were previously assigned to the *Pc-G* are also required for maintenance of activation at homeotic loci. Those proteins are now classified in the new enhancer of *trithorax* and *Polycomb* group (ETP) (Gildea et al., 2000).

In *Drosophila*, 15 *Pc-G* genes have been identified, although up to 40 *Pc-G* members may exist (Jürgens, 1985; Landecker et al., 1994). Many PC-G share conserved protein–protein interaction domains with other chromatin proteins. Genetic experiments show that the *Pc-G* and *trx-G* act synergistically in a dose-dependent manner, since a *Pc-G* gene mutation can increase another *Pc-G* mutant phenotype, and conversely a *trx-G* mutant can suppress a *Pc-G* mutant phenotype (Kennison, 1995). Synergistic action of *Pc-G* products is supported by the colocalization of several PC-G on polytene chromosomes: PH (*polyhomeotic*), PC, and PCL (*Polycomblike*) colocalize at all sites (Zink and Paro, 1989; DeCamillis et al., 1992; Lonie et al., 1994; Strutt et al., 1997), whereas other PC-G overlap at some but not all common sites. For instance, PSC (*Posterior sex combs*) shares 70% of its binding sites with PC/PH/PCL (Martin and Adler, 1993). Biochemical experiments show that PC-G proteins form large multimeric complexes and there is clear

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doi:10.1016/S0012-1606(03)00314-2

evidence for several distinct PC-G complexes (for review, see Simon and Tamkun, 2002). One of these, the *Polycomb* repressive complex 1 (PRC1) blocks chromatin remodeling mediated by the SWI/SNF complex, which contains *trx-G* members, and associates with general transcription factors such as TAFs (TBP-associated factors) and TBP (TATA-binding protein), suggesting relationships between PREs complexes and promoters (Shao et al., 1999; Breiling et al., 2001; Saurin et al., 2001). Therefore, a direct antagonism between PC-G and TRX-G proteins may be the basis of the mechanism of *Pc-G*- and *trx-G*-mediated regulation.

PC-G and TRX-G bind their target loci at specific DNA regions called *Pc-G* response elements (PREs) (Simon et al., 1993) and *trx-G* response elements (TREs) (Chan et al., 1994; Chang et al., 1995). Tillib et al. (1999) showed that there is overlap between TRE and PRE but that some motifs affected primarily one and not the other element. To reflect the dual function of regulatory elements that bind both groups of proteins, Brock and van Lohuizen (2001) proposed a new name to define homeotic PRE/TREs: the "maintenance elements" (MEs). Those regions share several features in vivo when they are present in a transgene (for review, see Kassis, 2002): (1) homeotic PREs maintain embryonic silencing of a reporter gene expression pattern initiated by homeotic parasegment-specific silencers (Simon et al., 1993; Chan et al., 1994; Chiang et al., 1995; Gindhart and Kaufman, 1995; Hagstrom et al., 1997; Mihaly et al., 1997; Shimell et al., 2000). (2) PREs induce repression of the adjacent *white* reporter gene, leading to a variegated phenotype (Fauvarque and Dura, 1993; Chan et al., 1994; Kassis, 1994; Pirrotta and Rastelli, 1994; Gindhart and Kaufman, 1995; Zink and Paro, 1995); (3) this variegated phenotype is dependent on the PC-G and TRX-G, since variegation is enhanced in a *trx-G* mutant and is suppressed in a *Pc-G* mutant background, while modifiers of position effect variegation (PEV) have no effect (Fauvarque and Dura, 1993; Fauvarque et al., 1995; Gindhart and Kaufman, 1995); (4) formaldehyde cross linking of chromatin and immunocytochemistry on polytene chromosome experiments demonstrates that PC-G are directly associated with PREs (Zink et al., 1991; Orlando and Paro, 1993; Chan et al., 1994; Chiang et al., 1995; Zink and Paro, 1995; Strutt et al., 1997; Strutt and Paro, 1997; Orlando et al., 1998); and (5) PREs induce pairing-sensitive repression (PSR) (Kassis et al., 1991; Fauvarque and Dura, 1993). Recent studies show that PRE/TREs are composed of several independent PC-G/TRX-G-binding modules that act synergistically to induce both embryonic silencing and/or pairing-sensitive repression (Tillib et al., 1999; Horard et al., 2000; Hodgson et al., 2001; Americo et al., 2002; Huang et al., 2002). Together, these recent data strongly suggest that each PRE/TRE is composed of multiple different *cis*-DNA modules, which can be bound by different subsets of PC-G and TRX-G at defined spatial and temporal positions in the embryo.

Only a few PC-G target loci have been studied. These include the *Antennapedia* (ANT-C) and the *bithorax* (BX-C) homeotic complex loci, the *engrailed/invected* locus (Dura and Ingham, 1988; Moazed and O'Farrell, 1992; Strutt and Paro, 1997; Americo et al., 2002), the *iroquois* complex (IRO-C) (Netter et al., 1998), and the *polyhomeotic* complex locus (Fauvarque and Dura, 1993; Fauvarque et al., 1995). Interestingly, these target loci are all organized in gene clusters, suggesting a possible chromatin-induced co-regulation of target genes within a defined locus.

In this study, we focused on the transcriptional regulation of a *Pc-G* member: the *polyhomeotic (ph)* gene. *ph* is a complex, tandemly repeated gene locus on the X chromosome, composed of two functional units: *polyhomeotic proximal (php)* and *polyhomeotic distal (phd)* (Deatrick et al., 1991). We have previously studied the wild type regulation of *ph* transcription using a *P{lacW}* transposon inserted within the *ph* locus in the ph^{lac+3} strain. In this *P* element, *lacZ* and *white* reporter genes are under the control of the endogenous *ph* regulatory elements (Fauvarque et al., 1995). *ph* regulation is dynamic and complex prior to germ band retraction, but after that stage, *ph* expression becomes ubiquitous (Fauvarque et al., 1995; Serrano et al., 1995; Hodgson et al., 1997). A 2.9-kb *cis*-DNA fragment from the *php* regulatory region induced variegation and pairing-sensitive repression of the mini*white* reporter gene in a *Pc-G*-dependent manner (Fauvarque and Dura, 1993). Interestingly, in vivo chromatin accessibility of this mini*white* gene correlates with gene silencing (Boivin and Dura, 1998).

Remarkably, genetic analysis of *ph* expression shows that the *Pc-G* gene *Posterior sex combs* (*Psc*) is required for activation, rather-than repression of *ph* (Fauvarque and Dura, 1995). This observation suggests that the *ph* PRE is different from all previously described PREs. In contrast to the homeotic genes, which are expressed strictly zygotically and exhibit spatially restricted expression patterns, *ph* is maternally and zygotically expressed, is spatially regulated in early embryogenesis, and is expressed ubiquitously later in development. Because *ph* regulation is *Pc-G*-dependent, this observation suggests that PC-G do not always silence target loci. Instead, PC-G may modulate expression of target loci. The observations that *ph* is activated by *Psc*, and not fully silenced by other PC-G, suggest that the *ph* PRE, and perhaps the TRE, are differently organized, or function differently, at the *ph* locus compared with homeotic loci. Analysis of a PRE/TRE from a locus whose regulation is different from homeotic loci should reveal new insights into PRE/TRE function, and the role of *Pc-G* and *trx-G* in gene regulation.

Here, we have carried out an extensive genetic and molecular analysis of *ph* PRE/TREs and characterized PC-G and TRX-G that act on those regions. We show using functional analysis and chromatin immunoprecipitation (ChIP) that the *ph* locus contains at least two PRE/TREs: a strong one in the proximal unit and a weaker one in the distal unit. The *ph* PRE/TRE acts at a short range on a flanking promoter, whereas homeotic MEs display long-

range silencing over several kilobases. The *php* PRE/TRE is modular and does not recapitulate the endogenous *ph* response to all *Pc-G* mutations, suggesting that locus structure or chromatin architecture is essential for function of the *ph* PRE/TREs. We discuss models to explain why the *ph* gene might be ubiquitously expressed, yet regulated by a PRE.

Materials and methods

Fly strains and constructs

All strains and crosses were maintained on standard culture medium at 25°C. Except where otherwise stated, alleles are described in Lindsley and Zimm (1992). Injections were performed by standard procedures (Spradling, 1986) into a w^{1118} strain. In some cases *P*-element insertions were mobilized by using the endogenous transposase insertion *P{ry*$^{+}$ *Δ2–3}* (99B), and new transformant lines were selected in a ph^{410} mutant background. Due to the large number of transgenic lines obtained for some constructs, we have preferentially chosen lines which presented a variegated eye phenotype for further genetic studies, since we have previously observed that line without variegated eye were insensitive to *Pc-G* and *trx-G* mutations. Analysis of transgenic fly eye color in *Pc-G* and *trx-G* mutant backgrounds was performed as described in Fauvarque and Dura (1993) and Fauvarque et al. (1995). For each construct, 6 or more independent transgenic insertions were analyzed. All the constructs are made in the pCaSpeR4 vector (Thummel and Pirrotta, 1992). Eye color analysis was performed at least twice on flies of the same sex and age. To avoid the potential effects from balancer chromosomes on eye color, comparisons were made with unbalanced trans-heterozygotes for each transgenic line. In Tables 1, 2, and 3, weak, moderate, and strong effects on *ph* expression as monitored by changes in eye color are indicated as +/++/+++ or −/−−/−−− for activating and repressing, respectively (see also Fig. 3). The coordinates, based on the *Drosophila* sequence (release 2.5) gb AE003423, of the fragments in the constructs described in Fig. 4 are as follows: *P{C4-418}* and *P{C4-418bis}*, 2.9 kb *Xba*I–*Pst*I (nt 72,935 to 70,027); *P{C4-811}*, 0.9 kb *Kpn*I–*Pst*I (nt 70,973 to 70,027); *P{C4-812}*, 2 kb *Xba*I–*Kpn*I (nt 72,935 to 70,973); *P{C4-813}* and *P{C4-813bis}*, 4.9 kb *Eco*RI–*Pst*I (nt 74,925 to 70,027); *P{C4-815}*, 15.5 kb *Sal*I–*Sal*I (nt 80,713 to 65,187); *P{C4-816}* and *P{C4-817}*, 4 kb *Pst*I–*Pst*I (nt 70,027 to 66,143); *P{C4-819}*, 6.8 kb *Xba*I–*Pst*I (nt 72,935 to 66,143); *P{C4-820}*, 4.7 kb *Eco*RI–*Eco*RI (nt 79,644 to 74,925); *P{C4-822}*, 0.9 kb *Xba*I–*Avr*II (nt 72,935 to 72,024); *P{C4-824}*, 1 kb *Avr*II–*Kpn*I (nt 72,024 to 70,973); *P{C4-825}*, 10.6 kb *Sal*I–*Kpn*I (nt 80,713 to 70,973); *P{C4-826}*, 2.3 kb *Xho*I–*Bam*HI (nt 60,771 to 58,448). *P{C4-827}* is a 1-kb PCR amplification product. *P{C4-830}* is a 6.8-kb construct which contains the *P{C4-418bis} Xba*I/*Pst*I fragment cloned just upstream of a 3.9-kb PCR amplification product of the bacterial *lacZ* gene. For simplicity, the coordinates described in the *phd* unit (*P{C4-826}*) are extrapolated from the other coordinates.

Quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated from 30 flies. Total RNA (4 μg) were treated with Amp. grade DNase (Invitrogen) for 20 min at room temperature. DNase was inactivated by incubating 15 min at 65°C. One-half of the RNA was reverse transcribed by using the Superscript II First-Strand Synthesis Kit (Invitrogen) according to the instructions supplied by the manufacturer. The other half was used as an RT minus control during the PCR. The *polyhomeotic proximal* transcript was amplified with 1/10 of the cDNA synthesized (2 μl) with 200 nmol of specific primers using the SYBR Green PCR Master Mix (Applied Biosystems) in a 50-μl reaction mixture. The product was amplified by 45 cycles of PCR (30 s at 95°C, 30 s at 60°C, and 45 s at 72°C), followed by the construction of a melting curve in order to verify the specificity of the PCR. The incorporation of the dye into the amplified products was monitored by iCycler (Bio-Rad), and the concentration of a specific transcript in the sample was analyzed by the associated software based on the standard curves predetermined with known amounts of target transcripts. Quantities of *rp49* gene transcripts were used as a total-cDNA input control. Results from at least three independent RT-PCR analyses were averaged. The DNA sequences of oligonucleotides used in RT-PCR analyses are: php F, 5′-TTG GGA GCC TCA ACA GAA GT-3′; php R, 5′-CCT AGA GGC ATT GCA AGA GG-3′; Rp49 F, 5′-AAC ATC GGT TAC GGA TCG AA-3′; Rp49 R, 5′-ACG TTG TGC ACC AGG AAC TT-3′.

Immunostaining of polytene chromosomes

Drosophila polytene chromosomes preparation and immunostaining were performed as previously described in Franke et al. (1992). The primary antibodies added to the preparations were a rabbit anti-PH (diluted 1/40), a rabbit anti-PC (diluted 1/75), and a mouse anti-PSC (mAb:diluted 1/10). For fluorescent detection of the primary antibodies, Cy3-conjugated anti-rabbit and anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratory) were used as secondary antibodies at dilutions of 1/500 and 1/250, respectively. Chromosomes were counterstained with DAPI (Sigma). The slides were mounted in Mowiol (Coger) mounting medium. Images of labeled chromosomes were acquired with a Leica microscope equipped with a digital camera and processed by using Adobe Photoshop software.

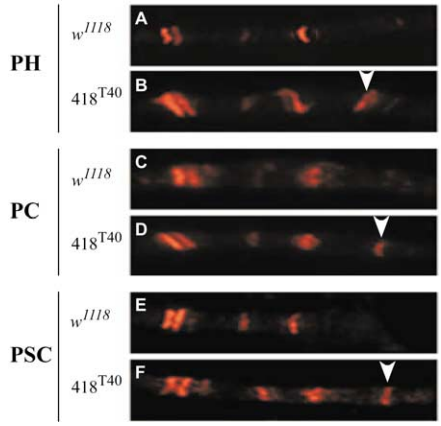


Fig. 1. *ph* PRE creates an additional site of PH, PC, and PSC immunostaining on polytene chromosomes. The w^{1118} 418^{T40} transgenic line contains a *P{418}* transposable element inserted in 9D on the chromosome X. In wild type w^{1118} X chromosomes, no endogenous PH, PC, or PSC protein binding is observed at location 9D (A, C, and E, respectively). In contrast, strong additional binding sites are observed in the w^{1118} 418^{T40} transgenic line at the insertion point of the *P{418}* transposon for PH, PC, and PSC (B, D, and F, respectively).

In vivo formaldehyde cross-linking of Schneider cells and embryos, and immunoprecipitation of cross-linked chromatin

Cross-linking of *Drosophila* Schneider cells and 11- to 16-h-old embryos and immunoprecipitation from cross-linked chromatin were carried out as previously described (Strutt et al., 1997; Cavalli et al., 1999). Purified DNA (100 ng) from antibody immunoprecipitations or control immunoprecipitation without antibody (Mock IP) was used as a hybridization probe on Southern blots, as previously described (Orlando and Paro, 1993; Orlando et al., 1997). Hybridization signals were quantified by using a PhosphorImager. Background hybridization taken from areas of the blots not containing any DNA was subtracted from the signal of each band. The resulting values were normalized by fragment size and then they were divided by the Mock signals to calculate relative enrichments. Enrichments were plotted along on the *y*-axis along with respect to the position of each fragment along the *polyhomeotic* locus.

Results

PC-G bind a transgene containing a putative PRE from the ph locus

We have previously shown that *ph* is quantitatively regulated in development, and that expression levels depend on mutations in *Pc-G* and *trx-G* genes (Fauvarque et al., 1995). Therefore, we wished to identify the PRE/TREs responsible for this regulation. We showed that a fragment, termed *P{418}*, that is located upstream of *php* and includes 50 bp downstream of the *php* transcription start (see also Fig. 2A), acts as a PRE in pairing-sensitive repression (PSR) assays, and exhibits variegated repression of *white* (Fauvarque and Dura, 1993). Because PC, PSC, and PH bind to the *ph* locus in polytene chromosomes (Zink and Paro, 1989; DeCamillis et al., 1992; Martin and Adler, 1993), we performed polytene chromosome immunostaining on the $P\{418\}^{T40}$ transgenic line. In $P\{418\}^{T40}$ line, the transgene maps at cytological location 9D in the X chromosome. No endogenous PH, PC, or PSC protein binding is observed at this location in wild type w^{1118} X chromosomes (Fig. 1A, C, and E, respectively). However, a strong additional binding site is observed in the $P\{418\}^{T40}$ transgenic line at the insertion point of the transposon for PH, PC, and PSC (Fig. 1B, D, and F, respectively). Therefore, this PRE is sufficient to recruit these proteins, meeting one of the five formal criteria for PREs discussed by Kassis (2002).

Functional analysis of the php PRE in transgenes

Polytene chromosome binding, PSR, and *white* repression assays have been used in the majority of functional tests of PREs in transgenic lines (for review, see Kassis, 2002). Because we wished to compare our results with as many previous studies as possible, we used the same assays to study *ph* PREs. We expanded our analysis of PRE function to test the role of various fragments in the *php* PRE. *P{C4-418}* and the same fragment in reverse orientation *P{C4-418bis}* behave identically in PSR and *white* repression assays when cloned into pCaSpeR4 vector (Fig. 2B, and Table 1). Therefore, this fragment functions in an orientation-independent manner.

We tried to identify the minimal sequence retaining PRE function. The minimal fragment that retains PSR, and exhibits variegated expression of *white*, is a 2-kb fragment *P{C4-812}*, that lacks the 3′ 900 bp of *418* (Fig. 2B). PSR and variegation were observed in about 45% of the *P{C4-812}* transgenic lines (Table 2), a frequency similar than the one previously published for the *418* lines (Fauvarque and Dura, 1993). We then tested two overlapping fragments of approximately 1 kb each (*P{C4-827}* and *P{C4-824}*; Fig. 2B), but neither of these fragments demonstrate PRE activity (Table 2). We also tested a fragment *P{C4-822}*, that has a 5′ deletion relative to *418*, but this fragment lacks significant PRE activity (Fig. 2B, and Table 2). *P{C4-811}* also lacks PRE activity (Fig. 2B, and Table 2). For *P{C4-827}*, *P{C4-824}*, *P{C4-822}*, and *P{C4-811}*, of at least six lines tested per construct, none showed PSR and variegation. Together, the results suggest that PRE activity requires overlapping sequences present in the *P{C4-812}* fragment.

We then tested the possible contribution of regions further upstream. We examined *P{C4-813}* and *P{C4-813bis}*, the same fragment in the opposite orientation, that contains an additional 2 kb upstream of *418* (Fig. 2B). As shown in Table 2, both these fragments exhibit repression of *white*, but the *813* fragments exhibits stronger PSR than *418*. We

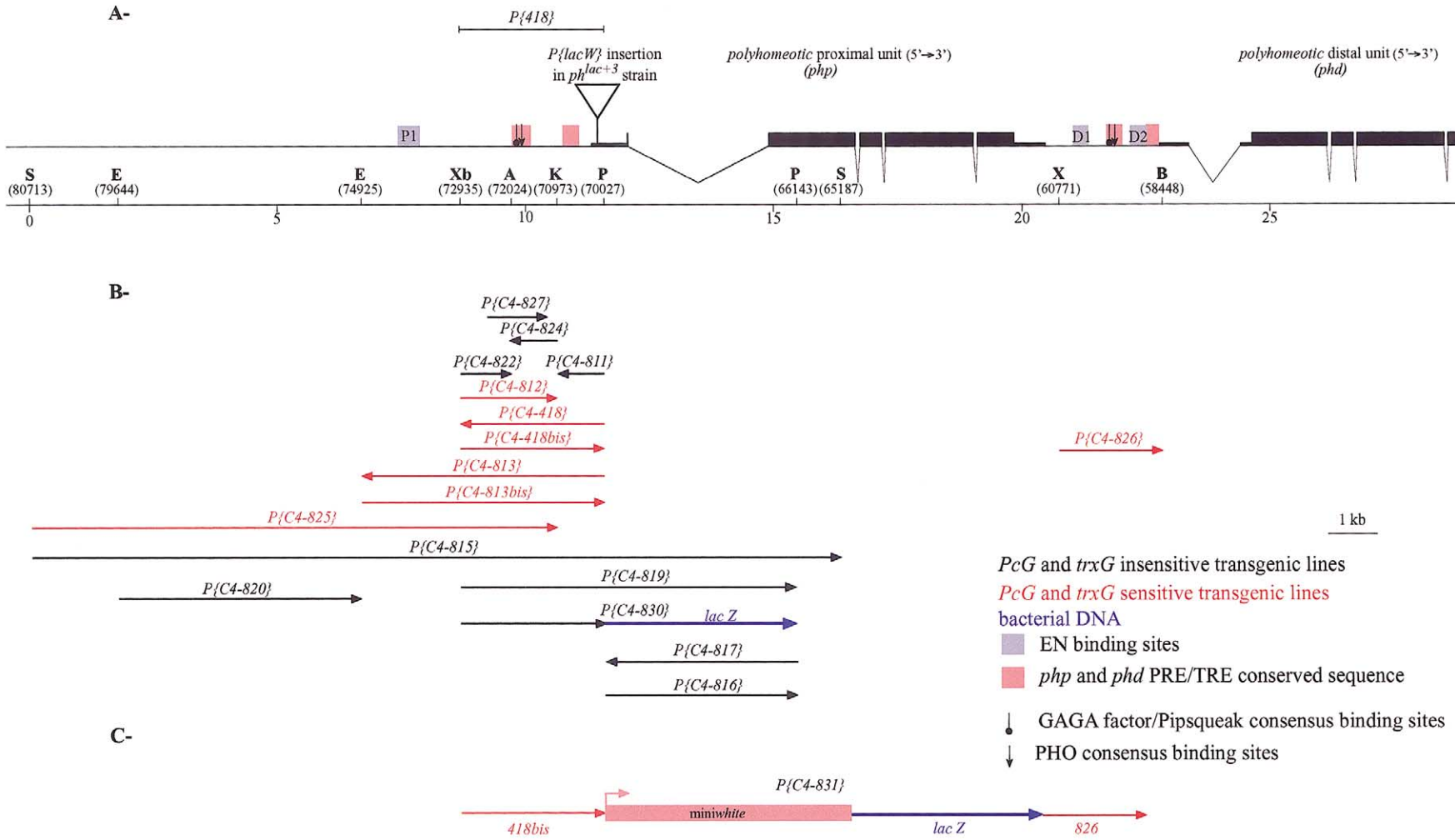


Fig. 2. *polyhomeotic* locus organization and constructs. (A) The *polyhomeotic* complex locus is composed of two units *polyhomeotic proximal (php)* and *polyhomeotic distal (phd)* localized on the chromosome X at cytological location 2D. Genomic DNA is presented as a thin line, and the exon structure of the two *ph* units is shown as a thick line. Restriction enzymes used for cloning are represented bellow: *Eco*RI (E), *Xba*I (Xb), *Avr*II (A), *Kpn*I (K), *Pst*I (P), *Sal*I (S), *Bam*HI (B), and *Xho*I (X). The restriction enzyme coordinates, based on the *Drosophila* sequence gb AE003423, are shown in brackets. EN, GAF/Pipsqueak, and PHO sites are respectively described in Serrano and Maschat (1998), Farkas et al. (1994), Hogdson et al. (2001) and Mihaly et al. (1998). (B) Schematic representation of DNA fragments studied in transgenic assays. The arrowhead indicates the $5' > 3'$ orientation of the mini*white* reporter gene in the pCaSpeR4 transformation vector and its location right or left with respect to the *ph* fragment being tested. (C) Schematic representation of the *P{C4-831}* construct. The *P{C4-831}* construct mimics the endogenous *ph* locus organization. This construct contains the mini*white* reporter gene surrounded by the *418bis php* and the *826 phd* PREs, as well as the *lacZ* gene inserted between the $3'$ end of mini*white* and the $5'$ end of the *826 phd* PRE to mimic the endogenous distance between the two PREs.

Table 1
Genetic comparison between *polyhomeotic* wild type regulation and the isolated *php* PRE/TRE

			Transgenic lines		
			Endogenous wild type regulation (ph^{lac+3}) (Fauvarque et al., 1995)	*P{C4-418}* *P{C4-418bis}*	*pCaSpeR4*
		Number of lines tested	1	20	6
		Variegation	1 (weak)	9	0
		Pairing-sensitive repression	0	9	0
		Eye color index			
		♂	6	4	5
		♀	5	2.5	4.5
	Gene	Allele			
PcG mutants	*polyhomeotic*	ph^{410}	+++	+++	nc
	Polycomb	Pc^{k}	++	−−	nc
	Sex comb extra	Sce^{1}	+++	−−−	nc
	Enhancer of Polycomb	*E(Pc)*	++	++	nc
	Posterior sex comb	Psc^{1} (gain of function)	+++	+++	nc
		Psc^{Arp1} (null)	−−	−−	nc
trxG mutants	*trithorax*	*Df(3R)red31* or *trx*	−−−	nc	nc
	brahma	*Df(3L)th102*	−−−	−−−	nc
	moira	mor^{1}	−−	−−−	nc
	kismet	kis^{2}	−−−	−−−	nc
	abnormal, small or homeotic disc 2	*Df(3R)XS*	−−−	−−−	nc

Note. For Table nomenclature refer to the legend of Fig. 3.

The eye color index refer to an arbitrary scale of eye color of heterozygous flies ranging from 1 to 7:0 indicates a white eye while 7 a wild type red eye, 1, 2, 3, 4, 5, 6 refer to pale yellow, yellow, pale orange, orange, pale red, red, respectively. The numbers shown in this table represent the average of all transgenic heterozygous lines for each construct for both males and females.

hypothesize that sequences upstream of *418* contribute to PSR, although this was not tested directly. To determine whether regions further 5′ contribute to PRE activity, we tested *P{C4-820}* (Fig. 2B). No PRE activity was observed in fragment *820*, showing that this fragment is not sufficient for PRE activity (Table 2). To complete our analysis of the *ph* proximal region, we analyzed fragments from the *php* intron (*P{C4-817}* and *P{C4-816}*; Fig. 2B). Neither exhibited PRE activity by themselves (Table 2).

Together, these results show that *ph* PREs meet two more formal criteria for PRE activity, namely PSR and repression of *white* reporters in eye disks (Kassis, 2002).

The php PRE has a local action

Surprisingly, transgenic flies transformed with the *P{C4-815}* and the *P{C4-819}* constructs, that contain, respectively, 17-kb and the 6.9-kb fragments encompassing the *418* 2.9-kb region (Fig. 2B) show no variegation and no pairing repressive effect (Table 2). Both constructs contain the *418* PRE/TRE located about 4–5 kb upstream of the mini*white* promoter. This suggests that the *php* PRE/TRE-induced repression may not be able to act at a long distance, or that there are sequences that suppress PRE/TRE activity in these large constructs. Homeotic PREs can be located tens of kbs away from their cognate promoters, and they are able to silence a reporter gene located several kbs away in a transgene (Chan, 1994; Zink and Paro, 1995), and therefore act at long range. To functionally test the role of distance in silencing mediated by the *php* PRE, we made a new construct containing the 418 PRE/TRE fragment separated from the mini*white* promoter by a 4-kb fragment containing the *lacZ* gene of *Escherichia coli* as DNA spacer (*P{C4-830}*; Fig. 2B). This same bacterial sequence was previously shown to allow silencing mediated by elements containing PREs from the BX-C, the *Fab-7* (Zink and Paro, 1995), and the *bxd1.5* elements (Chan, 1994) as well as the *engrailed* PRE (Americo et al., 2002). As expected from the "distance" hypothesis, *P{C4-830}* transgenic fly lines showed no variegation and no pairing repressive effect (Table 2).

Moreover, lack of long distance silencing does not depend on transcription of the *lacZ* sequence. We compared the expression of *lacZ* in four transgenic lines that exhibit PRE-mediated repression of *miniwhite* with two nonsilencing constructs described above (*P{C4-830-13}* and *P{C4-830-38}*). The four silencing lines were: one *bxd1.5 Ubx-lacZ* line (Hodgson et al. 2001), and three lines containing *lacZ* between *Fab-7* and *miniwhite*; the *5F24 25,2* and the *5F3 22,51* lines (Zink and Paro, 1995), and a derivative of this line carrying an *hsGAL4* transgene on chromosome 3 (*5F 322,51; hs-Gal4*/+). Normalized to *rp49*, we found by RT-PCR analysis of adult flies, that in these transgenic

Table 2
Genetic interactions of transgenic lines containing *polyhomeotic* PRE/TREs in *PcG* and *trxG* mutant backgrounds

			Transgenic lines			
			P{C4-812}	*P{C4-813}* *P{C4-813bis}*	*P{C4-811}* *P{C4-815}* *P{C4-816}* *P{C4-817}* *P{C4-819}* *P{C4-820}* *P{C4-822}* *P{C4-824}* *P{C4-827}* *P{C4-830}*	*P{C4-826}*
		Number of lines tested	15	22	6-10 per line	15
		Variegation	7	12	0	6
		Pairing sensitive repression	7	12**	0	6**
		Eye color index				
		♂	5.5	4	5 to 7	5
		♀	5	3	5 to 6	5
	Gene	Allele				
PcG mutants	*polyhomeotic*	ph^{410}	+++	+++	nc	+++
	Polycomb	Pc^{k}	−−	−	nc	nc
	Sex comb extra	Sce^{1}	nc*	−−	nc	nc
	Enhancer of Polycomb	*E (Pc)*	++	++	nc	+
	Posterior sex comb	Psc^{1} (gain of function)	+++	+++	nc	+++
		Psc^{Arp1} (null)	−	−	nc	−−
trxG mutants	*trithorax*	*Df(3R) red31* or trx^{E2}	nc	−−	nc	nc
	brahma	*Df(3L)th102*	−−	−−	nc	−
	moira	mor^{1}	−−	−−	nc	−
	kismet	kis^{2}	−−	−−	nc	−
	abnormal, small, or homeotic disc 2	*Df(3R)XS*	nc	−−	nc	−

* 5 of 7 *P{C4-812}* lines are insensitive in a Sce^{1} mutant background, while one line exhibits a darker eye color and one line a lighter eye color.
**Some of those lines are white or nearly white when homozygous.
For Table nomenclature refer to the legends of Table 1 and Fig. 3.

lines, the *lacZ* gene is transcribed at similar levels in flies with (*bxd1.5 UbxlacZ, FLW-1, 5F3 22,51, 5F3 22,51; hs-Gal4*) and without (*P{C4-830-13}, P{C4-830-38}*) silencing of *miniwhite* (data not shown). These data show that *lacZ* expression, or its absence, does not explain the failure of *P{C4-830}* lines to silence. Together, these results support the conclusion that *ph* PRE/TREs have a local action.

The php PRE is also a TRE, and is modular

In adults, endogenous *ph* is activated by members of the *trx-G (trx, brm, mor, kis, ash-2*) and repressed by proteins of the PC-G [*ph, Pc, Sce, E(Pc)*] (see ph^{lac+3} strain, Table 1). As noted in the introduction, *Psc* is unusual because it acts as an activator rather than a repressor of *ph* (Fauvarque et al., 1995; see Fig. 3A, C, and D, and Table 1). The *P{418}* lines induced silencing and pairing sensitive effects of the downstream *white* reporter gene in ≈45% of the resulting transgenic flies (Fauvarque and Dura, 1993). *P{418}*-induced silencing is suppressed in a *ph* mutant background and is enhanced in a *Pc* mutant background (Fig. 3F, G, and J). Genetic analysis of three independent *P{418}* transgenic variegated lines ($P\{418\}^{T3}$, $P\{418\}^{T30}$, and $P\{418\}^{T40}$) and lines in which the *418* fragment was cloned into the pCaspeR4 vector in either orientation (*P{C4-418bis}* and *P{C4-418}*) showed that the *php* PRE is sensitive to *Pc-G* and *trx-G* mutations, and is therefore a TRE in addition to being a PRE (Table 1). These studies confirm and expand previous studies showing that the *ph* PRE/TRE meets a criterion for PRE/TRE activity, namely that function depends on *Pc-G* or *trx-G* genes (Fauvarque and Dura, 1993).

The functional analysis of transgenes suggests that no one fragment contributes all the PRE activity of the *php*, which is consistent with a modular structure. If the *php* PRE is modular, and different PC-G contribute to function; as is the case for homeotic (Tillib et al., 1999; Horard et al., 2000; Hodgson et al., 2001; Huang et al., 2002) and engrailed PREs (Americo et al., 2002), then various *php* transgenes should exhibit differential sensitivity to *Pc-G* mutations. Therefore, we tested PSR and *white* variegation of all our transgenes for their sensitivity to *Pc-G* and *trx-G* mutations. Two results are noteworthy: *P{C4-813}* and *P{C4-*

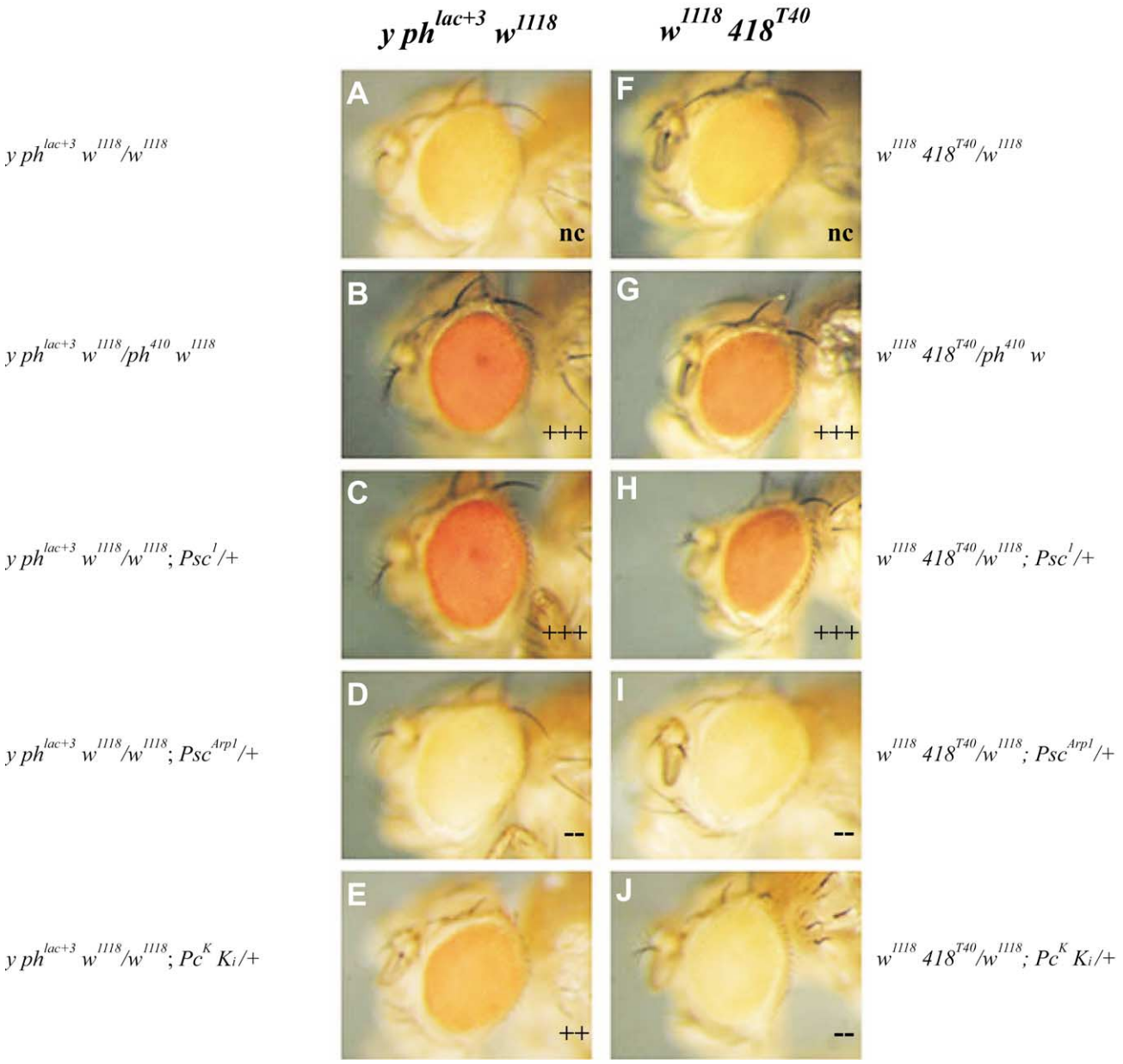


Fig. 3. *Pc-G* mutant eye color effects on endogenous *ph* PRE and transgenic *418 ph* PRE. Eye color of *y* ph^{lac+3} w^{1118}/w^{1118} and w^{1118} $418^{T40}/w^{1118}$ heterozygous females is orange in both cases (A and F, respectively). In a ph^{410} trans-heterozygous mutant background, mini*white* is strongly derepressed (B and G). *Psc* acts as an activator on *ph* transcription since, in a Psc^{Arp1} null allele, *white* expression is strongly decreased (D and I) while, in a Psc^{1} gain-of-function allele, *white* is strongly derepressed (C and H). Interestingly, in a *Pc* mutant background, *white* transcription in *y* ph^{lac+3} w^{1118} individuals appears partially derepressed (compare A and E), whereas in transgenic lines containing isolated PREs, *white* is strongly repressed (compare F and J). In the righthand corner of each panel, the level of expression of the *white* reporter gene in the eye is indicated by n.c. (no change in eye color), ++ or +++ (moderate or strong derepression: darker eye color) and −− or −− (moderate or strong repression: lighter eye color). This nomenclature is used in Tables 1, 2, and 3 to refer to the changes on *ph* expression in the assays described.

813bis} lines that contain the *418* fragment plus 2 kb upstream, including the EN (*engrailed*) *ph* proximal binding site (P1; see Fig. 2A), exhibit additional sensitivity to a *trx* mutant background compared with *418* or *812* (Table 2), suggesting that this region may contain a specific TRX response element. However, these 2 kb were not tested individually to determine whether they are sufficient for TRX response. The second interesting result is that *P{C4-812}* lines that do not contain 0.9 kb of downstream DNA found in the *418* fragment share sensitivity with *P{C4-418}* lines to all mutations except *Sce* and *ash-2* (Table 2). Therefore, we propose that SCE and ASH-2 response elements are contained in the 0.9-kb fragment. A model consistent with these results is shown in Fig. 6.

The php PRE/TRE does not completely recapitulate regulation of endogenous ph

In all cases where it has been tested, homeotic PREs behave similarly in transgenes compared with the endogenous locus. While this is partly true for the *php* PRE/TRE, some notable differences exist between *ph* wild type and regulation of isolated PREs in *Pc* and *Sce* mutant backgrounds (Table 1 and 2). In Pc^{k} and Sce^{l} mutant backgrounds, *white* is strongly repressed in *P{418}* transgenic lines (compare Fig. 3F and J) whereas it is derepressed in ph^{lac+3} (compare Fig. 3A and E). The simplest hypothesis to explain this divergence is that the *813*, *418*, and *812* minimal PRE, need additional sequences to respond correctly to *Pc* and *Sce* mutations. We then tested the *P{C4-825}* construct (Fig. 2B), which contains almost 10 kb upstream of the *php* promoter, including the *820* fragment that is bound by PH and GAF (see ChIP results below). Nevertheless, *P{C4-825}* transgenic lines exhibit the same differences in the genetic response to *Pc* and *Sce* mutations (data not shown), suggesting that lack of additional sequences does not explain the difference in behavior of transgenes relative to endogenous *ph*. Another possible explanation for these differences could be that, in the ph^{lac+3} transgenic line, the *P{lacW}* transposon is inserted at the endogenous *ph* locus. Therefore, the females analyzed in our crosses had two homologous copies of the endogenous *ph* sequences, which are located on the X chromosome. This was not the case for the isolated *P{418}* lines since the genetic assays were done in the heterozygous condition. Therefore, we performed the same eye color assays with an X-linked $P\{418\}^{T40}$ line in a homozygous condition. The same effects were observed in homozygous and heterozygous $P\{418\}^{T40}$ females in *Pc-G* and *trx-G* mutant backgrounds (data not shown). An alternative explanation for the different responses could be due to allele differences. We therefore assayed a group of *Pc* alleles to test for allele-specific effects. None of the *Pc* alleles tested that showed an effect exhibited the same effects on *ph* wild type regulation compared with the isolated $P\{418\}^{T40}$ PRE/TRE (Table 3). Interestingly, we note that *Pc* alleles that exhibit the strongest effects (Pc^{XL5} and Pc^{106}) have mutations in the chromodomain (Franke et al., 1995). Therefore, we conclude that the differences observed between *ph* endogenous regulation and the regulation of an isolated PRE/TRE are not due to differences in chromosomal pairing or to allele-specific effects.

Table 3
Genetic comparison between *polyhomeotic* wild type regulation and the $P\{418^{T40}\}$ isolated PRE/TRE using different *Polycomb* mutant alleles

Pc allele	*ph* wild type regulation	$P\{418^{T10}\}$ isolated PRE/TRE
Pc^{1}	nc	−−
Pc^{2}	nc	nc
Pc^{3}	+	−−
$Pc^{23937.4A}$	nc	−
$Pc^{23937.17B}$	+	−
$Pc^{23937.29A}$	++	−
$Pc^{23937.30A}$	nc	−
$Pc^{26865.4}$	nc	nc
$Pc^{29972.18}$	+	−
$Pc^{27385.C40}$	++	−
Pc^{106}	+++	−−
Pc^{K}	++	−
Pc^{Asx}	++	nc
Pc^{XM1}	+	−
Pc^{XL5}	+++	−−
Pc^{XT109}	nc	−

Note. Gray underline shows the two chromodomain affected *Pc* mutant alleles.

The ph locus contains two PREs

The *ph* locus is composed of two tandemly repeated transcriptional units (Deatrick et al., 1991) which correspond to the two *ph* genetic units (Boivin et al., 1999), that presumably arose by tandem duplication. To test whether the conserved nontranscribed region between the two *ph* transcription units contains a *phd* PRE/TRE, we tested the *P{C4-826}* construct (Fig. 2B). Transgenic *P{C4-826}* fly lines show strong pairing-sensitive repressive effects; however, sensitivity to *Pc-G* and *trx-G* mutations and eye color variegation are substantially weaker than in *php* PRE, except for *ph* and *Psc* mutants (Table 2). Nevertheless, these results show that the *ph* locus contains at least two similar PRE/TRE elements.

The association of proximal and distal ph PRE does not recapitulate wild-type ph regulation by PC

As mentioned above, mutations in the *Pc* gene induce a hyper-repression at the *ph* PRE when tested in transgenic constructs. This is in striking contrast to the effect of the same mutations at the endogenous *ph* locus, where *Pc* mutations derepressed both transcription of *ph* (by quantitative RT-PCR, see Discussion) and expression of the *white* reporter in the ph^{lac+3} strain. Moreover, this hyperrepression is never observed in transgenic constructs containing other known PREs or MEs. One possible explanation for the discrepancy between the effect of *Pc* mutations at the *ph* PRE in transgenes compared with the endogenous *ph* locus is that in this locus there are two PREs. These two PRE elements may act in a concerted manner to regulate the *ph* locus transcription, as the EN/EXD (*extradenticle*) complex may do for *ph* activation at earlier stages (Serrano and Maschat, 1998). For instance, looping interactions may bring the *php* PRE in physical contact of the *phd* PRE, and PC may be repressive in this context, while its action may be different in a different chromatin architecture, such as in transgenic constructs carrying the same PRE in isolation. To test whether *Pc* mediated repression at *ph* PRE requires the presence of both elements, we made the *P{C4-831}* con-

struct that mimics the endogenous *ph* locus organization (Fig. 2C). The *P{C4-831}* construct contains the mini*white* reporter gene surrounded by the 418bis *php* and the 826 *phd* PREs, as well as the *lacZ* gene inserted between the 3′ end of mini*white* and the 5′ end of the 826 *phd* PRE to mimic the endogenous distance between the two PREs (Fig. 2C). Three out of the four lines obtained exhibit mini*white* silencing, eye variegation, and PSR. However, both Pc^{k} and Pc^{XL5} mutations induced a strong hyperrepression of the mini*white* reporter (data not shown). Therefore, the particular arrangement of the *php* and the *phd* PREs is not sufficient to induce a repressive function for PC protein. This strongly suggests that other features of the *ph* locus allow silencing by the PC protein.

PH, PC, PSC, and GAF proteins bind to ph regulatory sequences in the endogenous ph locus

To begin our analysis of the molecular basis of *ph* regulation by PRE/TRES, we reasoned that important regulatory regions were likely to be conserved between the duplicated *php* and *phd* transcription units. Accordingly, we compared DNA sequences from upstream of the *php* start site, and the DNA sequence from the 3′ end of *php* to the transcription start of *phd*. Two conserved regions were found, each containing about 350 bp with 90% identity between proximal and distal *ph* units, and these are indicated as red blocks in Fig. 2A. One of these conserved regions contains canonical binding sites for the GAGA Factor (GAF) and PHO (*pleiohomeotic*) proteins. GAF was previously identified as a *trx-G* member (Farkas et al., 1994), but it has recently been shown to be also involved in PRE silencing function (Hagstrom et al., 1997; Strutt et al., 1997; Horard et al., 2000; Busturia et al., 2001; Hodgson et al., 2001). PHO is the only member of the *Pc-G* with sequence-specific binding activity (Brown et al., 1998; Mihaly et al., 1998). GAF and PHO sites are important for PRE function at other loci (Tillib et al., 1999; Fritsch et al., 1999; Shimell et al., 2000; Mishra et al., 2001; Busturia et al., 2001; Americo et al., 2002), so the presence of these sites in *ph* regulatory DNA is consistent with the existence of two PREs, one regulating *php* and the other regulating *phd*.

Formaldehyde cross-linking and chromatin immunoprecipitation (ChIP) experiments on known PC-G target genes have shown that these proteins bind preferentially in PRE-containing regions (Orlando and Paro, 1993; Strutt et al., 1997; Strutt and Paro, 1997; Orlando et al., 1998). Because the *ph* PRE(s) have not yet been defined molecularly, we analyzed the distribution of PC, PH, PSC (*Posterior sex combs*), and GAF proteins by ChIP on a *ph* genomic walk (Figs. 4 and 5). In *Drosophila* SL2 cultured cells, all four proteins are bound to regulatory regions of *ph* (Fig. 5B–E). Extensive colocalization was observed for all proteins, but it was particularly striking for PH and GAF proteins (compare Fig. 5C and E). It is notable that binding of these proteins is confined to about a 5-kb region upstream of *php*, and to the region between *php* and *phd*. A sharp decrease of binding to background levels was observed in the first intron of the *php* unit. The binding profile of PC and GAF was confirmed by analysis of staged embryos (Fig. 5F and G). An overlapping but not identical distribution was observed in embryos compared with cultured cells (compare Fig. 5F and B, and 5G and E). Additional binding peaks were detected about 4.9 kb upstream of the *php* transcription start corresponding to sequences located in the 5′ of the coding region of the *6-phosphogluconate dehydrogenase* gene. This indicates that PRE/TREs may regulate this gene as well.

Discussion

We have shown that the *ph* locus contains two PRE/TREs, that recruit PC-G proteins in vivo as shown by polytene staining of transgenes and ChIP analysis of the endogenous locus, and that transgenes containing the PRE/TREs exhibit PSR, repression of the *white* reporter in eye discs, and their activity is sensitive to mutations in *Pc-G* and *trx-G* genes. Thus, by these criteria, which have been used for the majority of PREs studies (Kassis, 2002), *ph* PRE/TREs are authentic. However, we were concerned that PSR and *white* repression assays of transgene activity might not reflect regulation of the endogenous *ph* locus. For the reasons below, we argue that the assays used here do correlate with endogenous regulation of *ph*. First, we have already shown that *ph* mutations affect steady state endogenous *ph* mRNA levels using in situ hybridization in embryos as an assay (Fauvarque et al., 1995), demonstrating that *ph* is regulated quantitatively in vivo. Secondly, in ph^{lac+3} embryos, in which a *white* gene is inserted in a *P* transposon near the *php* transcriptional start (see Fig. 2A), the *white* gene exhibits identical regulation to that of *php* itself (Fauvarque et al., 1995), arguing that expression of *white* faithfully reflects regulation of *ph*. Third, we examined the expression of *php* in wild-type and $Pc^{K}/+$ females using quantitative RT-PCR. We observed a 2.12 ± 0.05 ($n = 4$) increase in *php* expression in the $Pc^{K}/+$ mutants, showing that the endogenous *ph* locus and the transgenes used here are sensitive to *PcG* mutations. An important criterion of PRE activity is the ability to maintain spatially regulated embryonic silencing of homeotic genes. However, the ability of the *ph* PRE/TREs to maintain silencing of homeotic loci was not tested in our assays. Based on comparison of *ph* PREs to other PREs tested in the same assays, there are some structural and functional differences between the *ph* and homeotic PREs revealed by our analysis.

The php PRE/TRE is modular, but modules do not work independently

Our detailed analysis of the *php* PRE/TRE shows that this element is modular, and contains at least three regions of differential sensitivity to *Pc-G* and *trx-G* mutations (Fig.

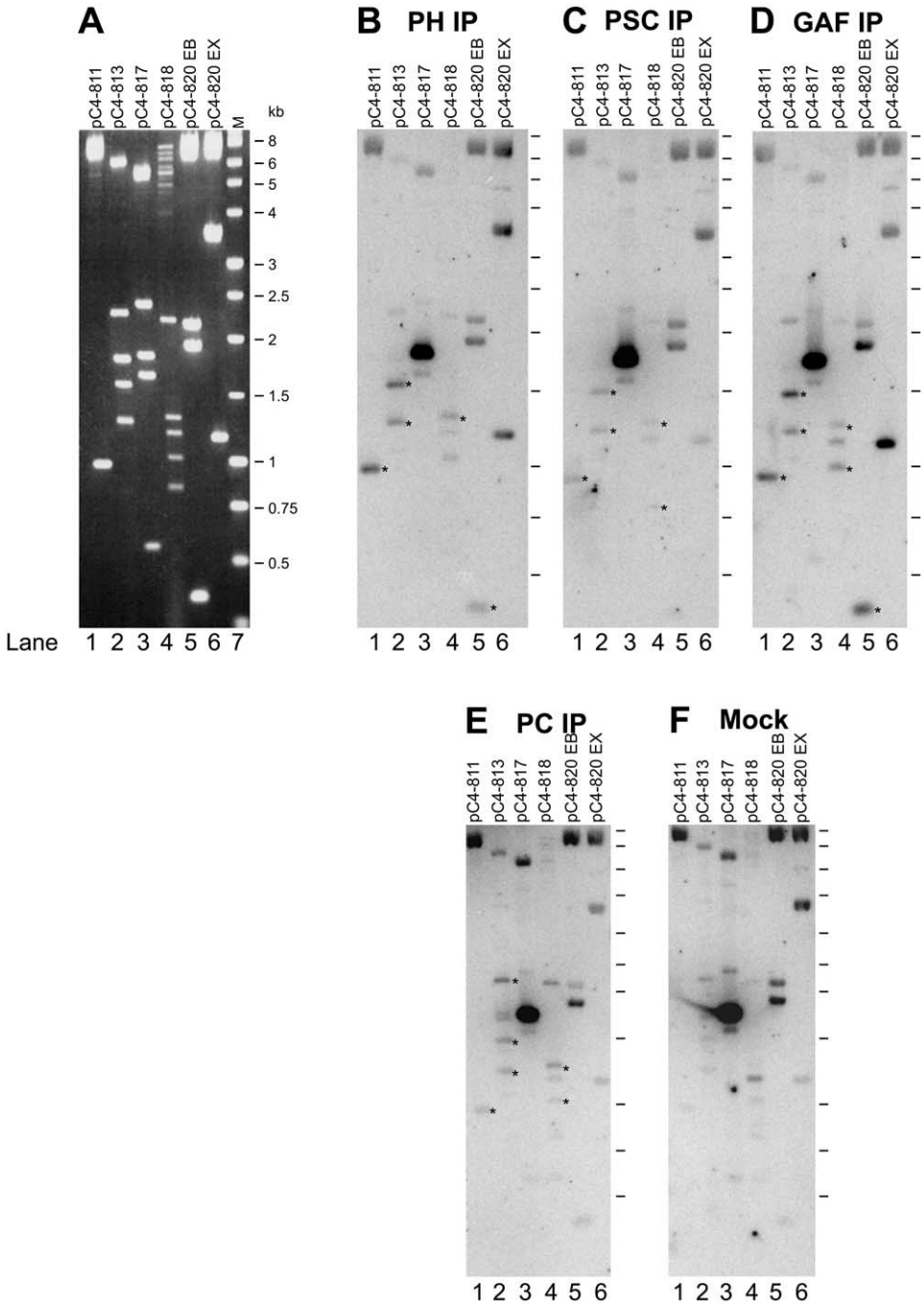
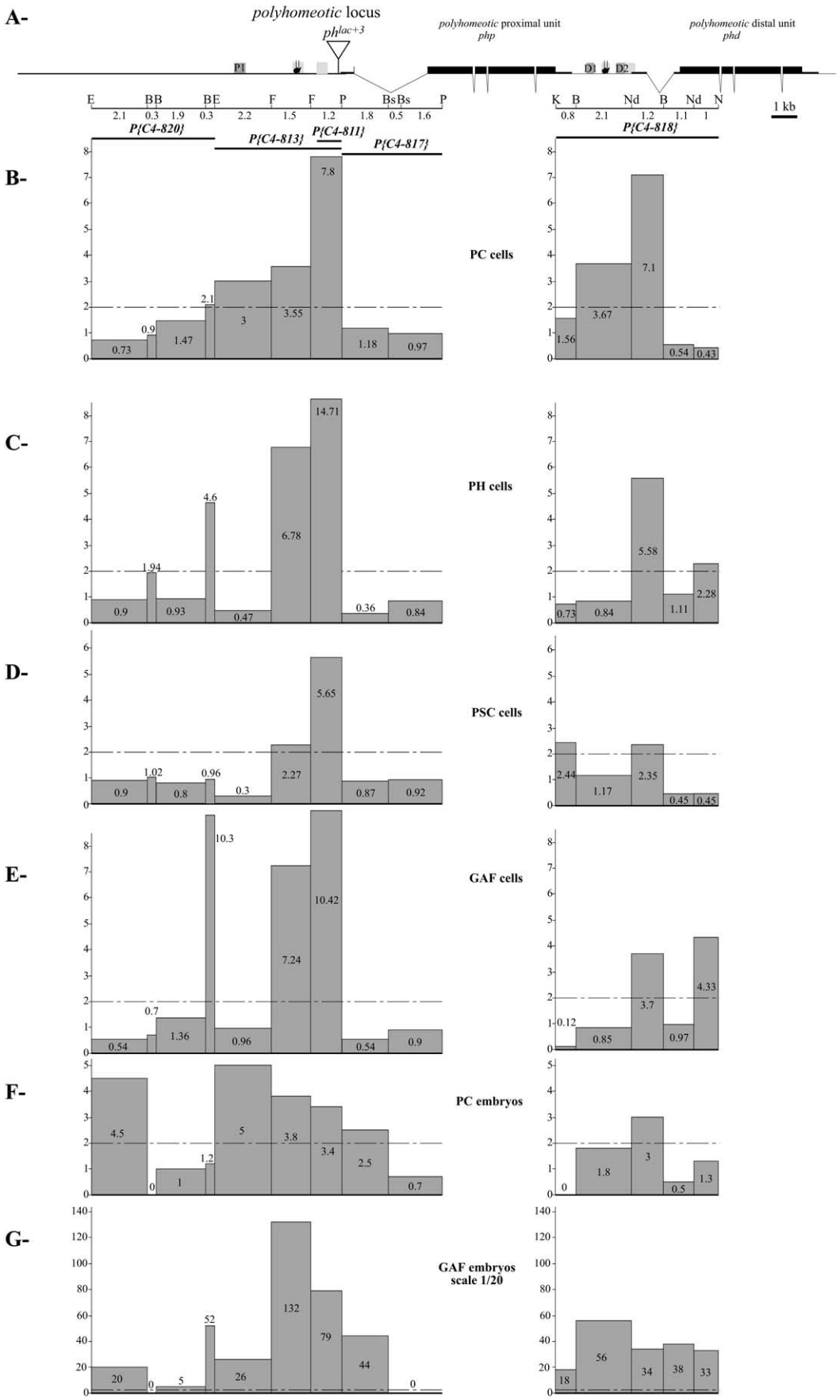


Fig. 4. Southern hybridization of immunoprecipitated DNA to the *polyhomeotic* complex genomic walk. ChIP experiments were performed with PH, PSC, GAF, and PC proteins in order to study their distribution along the *ph* genomic region. Subclones containing DNA fragments from the *polyhomeotic* locus (see Figs. 2A and 4B) were digested with restriction enzymes and separated on a 1% agarose gel. In (A–F), lane 1 is *P{C4-811}* digested with *Eco*RI and *Pst*I; lane 2 is *P{C4-813}* digested with *Eco*RI, *Pst*I, and *Fsp*I, lane 3 is *P{C4-817}* digested with *Pst*I and *Bxt*XI; lane 4 is *P{C4-818}* digested with *Bam*HI, *Kpn*I, and *Nde*I; lane 5 is *P{C4-820}* digested with *Eco*RI (E) and *Bam*HI (B); and lane 6 is *P{C4-820}* digested with *Eco*RI and *Xba*I. Lane 7 of (A), labeled as M, is a 1-kb ladder molecular weight (New England Biolabs). One representative ethidium bromide stained gel is shown in (A). In (B–F), Southern hybridization of similar gels using radioactive DNA probes obtained from ChIP experiments with antibodies against PH, PSC, GAF, and PC proteins in SL-2 *Drosophila* Schneider cells are shown. The immunoprecipitated protein is indicated above each panel and the size of molecular weight markers is shown by ticks on the righthand side of each panel. DNA fragments showing major enrichment compared to the Mock IP are indicated by dots flanking each band. The strong signal corresponding to the 1.85-kb fragment from *P{C4-817}* in the Mock IP (F) may be due to cross-hybridization to other portions of the genome.

6). Recently, it has been proposed that homeotic MEs are composed of several small DNA modules that are bound by several subsets of PC-G and TRX-G proteins (Tillib et al., 1999; Horard et al., 2000; Hodgson et al., 2001; Americo et al., 2002). In other PREs and MEs, the PSR is separable from silencing modules (Hodgson et al., 2001; Kassis,


A-
polyhomeotic locus
ph^lac+3
polyhomeotic proximal unit
php
polyhomeotic distal unit
phd
P1
D1
D2
E BB BE F F P BsBs P
2.1 0.3 1.9 0.3 2.2 1.5 1.2 1.8 0.5 1.6
K B Nd B Nd N
0.8 2.1 1.2 1.1 1
1 kb
P{C4-820}
P{C4-813}
P{C4-811}
P{C4-817}
P{C4-818}
B-
PC cells
0.73 0.9 1.47 2.1 3 3.55 7.8 1.18 0.97
1.56 3.67 7.1 0.54 0.43
C-
PH cells
0.9 1.94 0.93 4.6 0.47 6.78 14.71 0.36 0.84
0.73 0.84 5.58 1.11 2.28
D-
PSC cells
0.9 1.02 0.8 0.96 0.3 2.27 5.65 0.87 0.92
2.44 1.17 2.35 0.45 0.45
E-
GAF cells
0.54 0.7 1.36 10.3 0.96 7.24 10.42 0.54 0.9
0.12 0.85 3.7 0.97 4.33
F-
PC embryos
4.5 0 1 1.2 5 3.8 3.4 2.5 0.7
0 1.8 3 0.5 1.3
G-
GAF embryos
scale 1/20
20 0 5 52 26 132 79 44 0
18 56 34 38 33

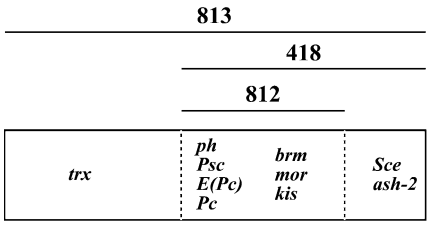


Fig. 6. Potential modular organization of the *php* PRE/TRE. Schematic summary of genetic results showing the potential modular organization of the *php* PRE/TRE. The minimum *php* PRE/TRE is contained in the *P{C4-812}* fragment since resulting transgenic files exhibit variegation, pairing sensitive repression and are sensitive to *ph, Psc, E(Pc), Pc, brm, mor,* and *kis* mutant backgrounds. Additional sensitivity is obtained with *Sce* and *ash-2* mutant background for *P{418}* transgenic flies. Further *trx* mutant sensitivity as well as additional silencing and PSR activities are obtained when *P{418}* is combined with an upstream 2-kb fragment in *P{C4-813}* transgenic lines. As far as we can tell, *P{C4-813}* corresponds to the complete *php* PRE/TRE.

2002). In our assays, *Pc-G*-dependent silencing and PSR are always linked in the *ph* PRE: both silencing and PSR are lost when the minimal 2-kb *P{C4-812}* PRE/TRE is dissected into several subfragments. We were unable to separate PSR from silencing of *white,* but this may be because our studies lacked sufficient resolution.

Unusually, we found that the smallest fragment retaining silencing and PSR activity is 2-kb. This is much larger than minimal fragments showing activity reported from previously described MEs or PREs. Reduced activity might be detected as a lower percentage of transgenic lines showing silencing or PSR, reduced strength of silencing or PSR, or both. As shown in Table 4, the *bxd* PRE, the *iab-7* PRE, and the *engrailed* PRE all exhibit detectable activity as 191-, 260-, and 181-bp fragments respectively. Because we examined at least three 1-kb fragments in the region showing PRE/TRE activity, it is unlikely that each fragment breaks in a key sequence that prevents activity. If so, there would have to be three key sequences contained within the *418* fragment, one for each unique breakpoint in the fragments tested (*822*, *824*, and *827*). Our results strongly suggest that the structure of the *php* PRE/TRE is different from previously characterized PREs or MEs.

There is not a perfect correspondence between sites of PC-G binding as detected by ChIP, and functional activity of transgenes. We mapped peak-binding for PC, PH, PSC, and GAF to a 0.9-kb fragment which showed no silencing activity and no sensitivity to *Pc-G* and *trx-G* mutations when tested in isolation in the *P{C4-811}* transgenic lines. In both cells and embryos, PC-G and GAF binding overlaps the conserved regulatory sequences found in both the *php* and *phd* PREs. However, neither of these conserved sequences were sufficient for PRE activity in our assays. The *P{C4-827}* and *P{C4-824}* fragments overlap, and both contain the upstream 350-bp conserved region, but neither demonstrate PRE activity. Similarly, *P{C4-811}* that contains the downstream conserved region also lacks PRE activity. We do not know why these differences exist; but suggest that proteins not assayed in the ChIP experiments, and/or unconserved sequences, must contribute to PRE function. In addition, there were differences between binding of PC-G proteins in cultured cells and in embryos, which may reflect the different physiology of developing embryos versus cultured cells or experimental variation of the technique.

Transgenes containing php PRE/TREs do not recapitulate endogenous ph regulation

The isolated *ph* PRE/TREs do not completely reproduce the *ph* wild type regulation even if most of the *Pc-G* and *trx-G* response elements are conserved in the minimal *P{C4-812}* PRE/TRE. We observe an opposite eye color phenotype in a *Sce* and *Pc* mutant background when the endogenous *ph* regulation is compared with transgenic lines containing isolated *ph* PRE/TREs. It may be simply that the effects of *Pc-G* mutations on *ph* regulation are indirect. It may be that different PC-G complexes function differently in the endogenous and exogenous chromosomal locations. Recently, using an in vivo-functional assay, Beuchle et al. (2001) showed that *ph* and *Psc* may have a different silencing function than *Pc* and *Sce*. Consistent with this, in our study, *ph* and *Psc* consistently show strong genetic effects on *ph* regulation. Our results are in accordance with the idea that *ph/Psc* in one hand and *Pc/Sce* in the other hand may play different roles in PC-G silencing complexes, and that the function of these complexes may depend on chromosomal context. An alternative, but not necessarily mutually exclusive explanation for the opposing response of *php* PRE/TRE transgenes and the endogenous *ph* locus to *Pc-G* mutations might be that the isolated PRE/TREs lack distant *cis*-DNA regulatory elements that can act on *ph* wild type regulation within the endogenous chromosomal context. In this model, activity of the *php* PRE/TRE would be modulated by the distant cis-regulatory element, so that transgenes, lacking this sequence would behave differently.

Fig. 5. Distribution of PC, PH, PSC, and GAF in the *polyhomeotic* complex. Quantitative representation of ChIP enrichments from Fig. 4 at the *ph* locus. (A) The *ph* locus is depicted in the proximal to distal orientation. The constructs *P{C4-820}, P{C4-813}, P{C4-811}, P{C4-817},* and *P{C4-818}* and a restriction map with the sites used in ChIP experiments are represented below the locus. *Eco*RI (E), *Bam*HI (B), *Pst*I (P), *Fsp*I (F), *Kpn*I (K), *Nde*I (Nd), *Bst*XI (Bs), *Not*I (N). Relative enrichments are indicated on the *y*-axis, while the position along the *ph* locus is indicated in the *x*-axis. Relative enrichments are shown for PC (B), PH (C), PSC (D), and GAF (E) from on SL-2 *Drosophila* Schneider cells, as well as PC (F) and GAF (G) from 11- to 16-h-old embryos. The dashed line in each panel represents the approximate background level. Enrichments below this value are not considered to be significant.

Table 4
Comparison of minimal PREs and MEs fragments that silenced mini*white*

PRE/ME	Description	Length (bp)	Variegation	PSR	References
engrailed	cf Kassis (1994)	181	yes	yes (62%)	Americo et al. (2002)
iab-7	*Apa*I–*Xba*I fragment	880	yes	yes (32%)	Mishra et al. (2001)
	Subfragment of the 880 bp PRE	260	yes	yes (22%)	Mishra et al. (2001)
bxd	*Eco*RI-*Sty*I fragment	1557	yes (54%)	yes	Chan et al. (1994)
	Subfragment of the 1557 bp PRE	661	yes (73%)	yes (>35%)	Sigrist and Pirrotta (1997)
	AB X 6	1836 (306 × 6)	no	yes	Horard et al. (2000)
	BP X 6	1146 (191 × 6)	yes (95%)	yes	
	BP X 1	191	yes (35%)	nd	
	PF X 4	308 (77 × 4)	yes	yes	
php	*812* fragment	1962	yes (47%)	yes (47%)	This study

These hypotheses cannot be distinguished with the current data.

The php PRE/TRE does not work at long range

The MEs of homeotic loci in Drosophila work at distances of tens of kilobases. Here, we show that the *php* PRE/TRE does not silence when separated from the promoter by a 4-kb *ph* intron, as in the *P{C4-815}* and *P{C4-819}* lines. The *418* fragment contains the endogenous *ph* promoter, as the 3′ end of the fragment is 50 bp downstream of the transcription start site as inferred from analysis of the *php* cDNA, and the *418* fragment promotes expression of *lacZ* (Fauvarque and Dura, 1993). One possibility is that the *ph* promoter prevents interaction of the *418* fragment with the distal *white* promoter, when the *418* fragment is separated from the *white* promoter by 4 kb.

Another possibility is that *php* sequences downstream to the PRE may contain enhancer elements counteracting the PRE or a chromatin insulator element (West, 2002) that may prevent spreading of silencing to distal regions. Alternatively, the downstream sequences could contain a promoter and a truncated-transcription unit that could be spliced. These possibilities were eliminated by showing that, when the bacterial *lacZ* gene that permits homeotic PRE function was inserted between the *418* fragment and the *white* gene, silencing by the *ph* PRE/TRE is abolished. While sequences in the *php* intron may interfere with *418* PRE activity, the *lacZ* sequence has already been demonstrated to be free of sequences that interfere with homeotic PRE-mediated silencing (Chan et al., 1994; Cavalli and Paro, 1998; Hodgson et al., 2001; Americo et al., 2002). Therefore, the simplest explanation is that silencing induced by the *php* PRE/TRE is determined by a short-range repression mechanism and that silencing cannot spread over long distances. This result is consistent with the ChIP binding studies showing that PC-G proteins do not spread past the *php* and *phd* PRE/TREs. The ability of this PRE to silence a reporter gene only when it is close to the PRE but not when it is located 4 kb away may depend on the inability of PC-G to spread across large distances from the *ph* PRE.

Models of php PRE/TRE function

The fact that *ph* is maternally and ubiquitously expressed yet regulated by *Pc-G* genes, raises the question of how the *php* PRE/TREs function. Previous studies suggest that PC-G and TRX-G act at the *ph* locus by modulating the *ph* transcription level, rather than by silencing (Fauvarque et al., 1995). *ph* is likely not the only gene that is modulated by the PC-G and TRX-G. One example of this type of regulation concerns the gene *toutatis (tou)*, a newly defined *trx-G* gene (Fauvarque et al., 2001). The location of this gene corresponds to the transcript VI described by Strutt and Paro (1997). The *tou* transcript is strongly and ubiquitously expressed throughout development, the *tou* locus is bound by PH and PSC proteins and is quantitatively misexpressed in *ph* mutants (Strutt and Paro, 1997). *ph* and *tou* may be the first members of a novel class of PC-G and TRX-G target genes that may be modulated instead of repressed in an all or none fashion.

One common feature of genes regulated quantitatively by PC-G and TRX-G may be that their PRE/TREs are located close to promoters. These PRE/TREs may have a short-range action depending on a direct contribution of PC-G and TRX-G complexes to the efficiency of the transcription machinery assembly on the promoter. On the other hand, PRE/TREs located far away from promoters and enhancers may depend on distant interactions that stabilize long-range architecture.

The regulation of *ph* by its PRE/TREs is apparently different from regulation of homeotic loci by PREs, because *ph* expression is ubiquitous, whereas homeotic loci are silenced, at least in some portions of the embryo. Nevertheless, *ph* and homeotic PREs may have some features in common. The *bxd* PRE does silence *Ubx* expression in anterior parasegments, but it also acts within parasegments to modulate *Ubx* expression. In wild type embryos, there is an anterior gradient of *Ubx* expression within parasegments from anterior high to posterior low. There are also variations in *Ubx* expression levels between parasegments, as expression of *Ubx* is highest in parasegment 6, but lower in more posterior parasegments. Both the variation of *Ubx* expres-

sion within and between parasegments is abolished in embryos mutant for *esc*, *Pc*, or *ph* (see for example, Fig. 1, McKeon and Brock, 1991), arguing that *Pc-G* genes, and by implication the *bxd* PRE, also has a role in modulation of homeotic gene expression. One could argue that the effects of *Pc-G* mutations on *Ubx* expression are indirect. However, we have evidence from studies of *bxd* transgene expression in embryos that small fragments of the *bxd* PRE modulate levels of expression as well as spatial restriction of expression of reporter genes (B. Argiropoulos, J.W. Hodgson, and H. W.B, unpublished observations). Therefore, we argue that, depending on the cellular context, homeotic PREs can silence in anterior parasegments, or modulate gene expression in posterior parasegments. We have not directly tested whether the ph PRE/TRE can silence in transgenes containing homeotic loci, or whether homeotic PREs can modulate gene expression. However, in recent work, Czermin et al. (2002) showed that *ph* and *Psc/Su(z)2* loci (2D and 49F, respectively) are strongly bound, on polytene chromosomes, by PSC but only weakly by an antibody anti-trimethylated lysine 9 of histone H3 (H3me3K9). Interestingly, PSC and H3meK9 antibodies colocalized at most PC-G binding sites, including the BX-C and ANT-C loci. This result suggests that PC-G-silenced loci are H3me3K9, whereas PC-G nonsilenced target are not. It will be interesting to determine whether the *ph* PRE differs from that of homeotic PREs because it does not silence, and whether the differences can be correlated to histone methylation. Taking our results together, we propose that modulation of gene expression is part of the normal repertoire of PREs. Is it possible that *ph* PREs are simply weak PREs compared with homeotic PRES, because they reduce rather than silence expression? Where it is possible to make direct comparisons between *ph* PREs and homeotic PREs in the same assays (PSR and *white* repression), *ph* PREs act similarly to homeotic PREs in terms of number of lines exhibiting variegating expression, the strength of the variegation, and the magnitude of responses to *Pc-G* or *trx-G* mutations (see Table 4), arguing that *ph* PREs are not weaker than homeotic PREs, at least in these assays.

One could imagine that the mechanism of modulation by PREs is similar to silencing, but based on the number, type and position of binding sites present, coupled with local differences in concentration of PRE binding factors, complete repression cannot be established. Understanding how PC-G proteins act at different PREs is a key challenge for future research.

Acknowledgments

We thank Renato Paro for the generous gifts of several ChIP probes. We thank Paul Adler, Kathy Matthews at the Indiana Drosophila Stock Center, and Renato Paro for fly stocks. We thank Keith Humphries and Corrina Auld for their help with real-time quantitative RT-PCR and Siegfried Bozza for injection and screening of the *P{C4-831}* line. We thank Leonie Ringrose and Jacob Hodgson for stimulating discussion and critical comments on the manuscript. S.B. was supported by a fellowship from the Ministère de l'Education Nationale and by a grant from the Association pour la Recherche sur le Cancer. The work of G.C. was supported by grants from the Centre National de la Recherche Scientifique (ATIPE), the Association pour la Recherche sur le Cancer (n.°5582), the Human Frontier Science Program Organisation, and the FSER Foundation. H.W.B. was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. The work of J.M.D. was supported by grants from the Indo French Centre for the Promotion of Advanced Research (Project n° 1603-1), the Centre National de la Recherche Scientifique (ATIPE n°7), the Association pour la Recherche sur le Cancer (n°5658), the Fondation pour la Recherche Médicale, and also from the University Paris XI, Orsay (UPRES-A Q8080), where this work was initiated at the Laboratoire d'Embryologie Moléculaire.

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