Modulation of the Activity of a Polycomb-Group Response Element in Drosophila by a Mutation in the Transcriptional Activator Woc

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ABSTRACT Polycomb group response elements (PRE) are cis-regulatory elements that bind Polycomb group proteins. We are studying a 181-bp PRE from the Drosophila engrailed gene. This PRE causes pairing-sensitive silencing of mini-white in transgenes. Here we show that the 181-bp PRE also represses mini-white expression in flies with only one copy of the transgene. To isolate mutations that alter the activity of the 181-bp PRE, we screened for dominant suppressors of PRE-mediated mini-white repression. Dominant suppressors of mini-white repression were rare; we recovered only nine mutations out of 68,274 progeny screened. Two of the nine mutations isolated are due to the same single amino acid change in the transcriptional activator Woc (without children). Reversion experiments show that these are dominant gain-of-function mutations in woc. We suggest that Woc can interfere with the activity of the PRE. Our data have implications for how Polycomb group proteins act to either partially repress or completely silence their target genes.

Polycomb group genes (PcG) encode proteins that mediate transcriptional repression. First identified in Drosophila as genes necessary to maintain the silencing of homeotic genes, it is now evident that PcG proteins have many other targets (reviewed in Simon and Kingston 2007). In genome-wide studies, PREs were identified in DNA elements that recruit PcG proteins to the DNA (reviewed in Schwartz and Hagstrom 1997). In the other assay, PREs are used to repress expression of the mini-white reporter gene in transgenic flies. Because mini-white repression is stronger in flies homozygous for the PRE-mini-white reporter, this latter assay has been called pairing-sensitive silencing (Kassis 1994).

One of the puzzles of the transgene assays for PREs is that silencing does not occur at every chromosomal insertion site. For example, for the four engrailed and invected PREs, pairing-sensitive silencing was observed at a frequency of 21–62% of insertion sites (Americo et al. 2002; Cunningham et al. 2010). PRE activity is regulated by the expression state of the gene it regulates; thus it follows that PRE activity in transgenes is dependent on the activity of regulatory elements that flank the transgene insertion site.

We have been studying a 181-bp en DNA fragment that acts as a PRE in several different assays: (1) it represses inappropriate expression in both en- and Ubx-reporter genes in embryos (Americo et al. 2002; Devido et al. 2008); (2) PcG proteins are associated with it in tissue culture cells, embryos, larvae, and adults (Strutt and Paro 1997; Nègre et al. 2006; Oktaba et al. 2008); and (3) it acts as a pairing-sensitive silencing element (Kassis 1994). This fragment contains...
binding sites for the PRE DNA binding proteins Pho, Pho-like, GAGA factor, and Spps (Americo et al. 2002; Brown et al. 2005; Brown and Kassis 2010). Thus, the 181-bp DNA fragment is clearly a PRE. Therefore, we reasoned that conducting a genetic screen for mutations that alter the activity of this PRE might yield mutations in PcG genes.

We conducted a genetic screen for dominant suppressors of pairing-sensitive silencing by a transgene that contained the 181-bp en PRE and mini-\textit{white}. These mutations were rare; we only obtained nine suppressors among 68,274 genomes screened. None of the mutations affected mini-\textit{white} repression of transgenes at all chromosomal insertion sites. This suggests that none of the mutations affects PRE activity directly. Instead, we believe that these mutations affect the expression of genes flanking the transgene insertion site. Consistent with this, two of the dominant suppressors are the same gain-of-function mutation in the gene \textit{without children} (\textit{woc}), which encodes a transcriptional activator. Our data suggest that there is a competition between transcriptional activators and PcG repression and that certain types of activators may be better able to overcome PcG repression.

**MATERIALS AND METHODS**

**Mutagenesis**

For EMS mutagenesis, adult males were fed EMS as described (Lewis and Bacher 1968; Kennison 1983), and then discarded 3–4 days following treatment to avoid pre-meiotic clusters of mutations. For the X-ray mutagenesis, males were irradiated with 30–40 Gy at 120 keV using a Faxitron Torrex 2800. The irradiated males were discarded 4–5 days following treatment.

**Sequencing**

DNA was isolated from homozygous or hemizygous mutant adults or larvae, and the entire \textit{woc} transcription unit was sequenced.

**Construction of P[\textit{L181PRE}]**

The 181-bp \textit{en} PRE was amplified with the primers GCGGAATTCGAGATGGAATGACGACTTTGCTGTAGCAG, cut with EcoRI, and cloned into EcoRI cut, phosphatased EK710, which contains \textit{loxP} sites on both sides of the EcoRI site (Kuhn et al. 2004). A fragment of DNA containing the 181-bp PRE and flanking \textit{loxP} sites was cut with NotI and cloned into NotI cut \textit{CaSpeR4}. The resulting clone was sequenced to determine the orientation of the insert.

**Generation and analysis of transgenic lines**

\textit{P[\textit{L181PRE}]} was injected into homozygous \textit{Df(1)w67c23}, \textit{y} embryos using standard techniques. Some lines were generated by \textit{P}-element mobilization by crossing to a strain with the endogenous transposase insertion \textit{P[\textit{ry} \Delta 2, 3]99B} (Robertson et al. 1988). \textit{P/[\textit{L}]I} derivative lines lacking the \textit{en181bp}-PRE were obtained by crossing males with the \textit{P[\textit{L181PRE}] insertion to virgin females that carried a constitutively active \textit{Cre} recombinase transgene (\textit{y}; \textit{CyO}, \textit{P[\textit{ Crew}]/\textit{ Sco}}) (Siegal and Hartl 1996). Progeny that contained both \textit{P[\textit{L181PRE}] and \textit{CyO}, \textit{P[\textit{w+w}:\textit{Cre}]} were crossed to \textit{Df(1)w67c23}, \textit{y} flies. Two individual \textit{w\textsuperscript{+}} male progeny were selected from each insertion line and crossed to the appropriate balancer chromosome. \textit{P/[\textit{L}]} lines were established, and the deletion of the \textit{en181bp-PRE} was confirmed by PCR with primers flanking the \textit{loxP} sites.

**qRT-PCR**

Fly lines of the following genotypes were used: (1) \textit{w\textsuperscript{1118}}, (2) \textit{w\textsuperscript{1118}}, \textit{P[\textit{L181PRE]}\texttt{8-10C}}, (3) \textit{w\textsuperscript{1118}}, \textit{woc\textsuperscript{D1}}, and (4) \textit{w\textsuperscript{1118}}, \textit{P[\textit{L181PRE]}\texttt{8-10C}}, \textit{woc\textsuperscript{D1}}. Total RNA from 3\textsuperscript{rd} instar larvae, 1-day-old pupae, or adult flies was prepared (Lorenz et al. 1989) and treated with DNase I before use. qRT-PCR was done with the QuantiTect SYBR Green RT-PCR kit (Qiagen) on the LightCycler 480 real-time PCR system (Roche Applied Sciences) using 0.2 μg total RNA/reaction. The following PCR primers were used: for the \textit{BpL32} reference gene, CGGATGATGATTAGCTGTT and CGGGGAATTCGCATGCTGGAGCTGTCC, its amplicon is 67 bp; for \textit{Ct30456}, AAAATGGCAGAACTTCTC and AACCTGGCCACCAATGCTG, its amplicon is 95 bp; for \textit{GstS1}, GTCAGAGCAACGATGTCACA and GGTGATGCTGGGAGTAG, its amplicon is 72 bp. Reverse transcription was done at 50° for 20 min, followed by incubation at 95° for 15 min to activate the PCR reaction. PCR was for 45 cycles of 94°, 10°, 60° 20°, 72°, 20°. After PCR, the reactions were heated to 95° and then cooled to 40° to analyze the melting temperatures of the PCR products.

**RESULTS**

**Dominant modifiers of mini-white repression**

To recover mutations that affect pairing-sensitive silencing, we screened for dominant mutations that suppressed \textit{en181bp}-mediated mini-\textit{white} repression. We used the line \textit{P[\textit{181PRE]}\texttt{8-10C}}, which contains a P-construct with the 181-bp PRE of \textit{en} DNA cloned into \textit{pCaSpeR} (Construct 8 in Kassis 1994; Figure 1). \textit{pCaSpeR} contains the mini-\textit{white} gene; a truncated version of the \textit{white} gene, which contains a promoter fragment that gives expression in the eye but no eye enhancer. The 181-bp PRE is cloned directly adjacent to the mini-\textit{white} promoter. The \textit{w}; \textit{P[\textit{181PRE]}\texttt{8-10C}} homozygotes have white eyes, and \textit{w}; \textit{P[\textit{181PRE]}\texttt{8-10C}} heterozygotes have orange eyes (Figure 2). For the mutagenesis, we fed \textit{w}; \textit{P[\textit{181PRE]}\texttt{8-10C}} males EMS and crossed to either \textit{w}; \textit{P[\textit{181PRE]}\texttt{8-10C}} or \textit{w}; \textit{P[\textit{181PRE]}\texttt{8-10C}} \textit{Sco/CyO} females. We looked for mutations that darkened the eye color of either homozygotes or heterozygotes. We recovered nine mutations; one on the X chromosome, four on chromosome 2, and four on chromosome 3. All but two of the mutations darkened the eye color of both \textit{P[\textit{181PRE]}\texttt{8-10C}} homozygotes and heterozygotes. These mutations could identify genes involved in repression of mini-\textit{white} transcription, perhaps via the PRE. One second-chromosome mutation darkened the eye color of heterozygotes only, which suggests that it is not involved in mini-\textit{white} repression but might be involved in pigmentation. We did not study this mutation further. The sex-linked mutation only darkens the eye color of \textit{P[\textit{181PRE]}\texttt{8-10C}} homozygotes. The reason for this is unknown; however, it could mean that the mutation affects the interaction between PREs.

**Two mutations cause the same single amino acid change in the transcriptional activator \textit{Woc}**

We mapped the mutations on chromosome 3 using the markers \textit{ru}, \textit{h}, \textit{th}, \textit{cu}, \textit{sr}, \textit{co}, and \textit{Pr}. Two mutations mapped 2.5 map units distal to \textit{Pr}. We next tried to recover recombinants between these two mutations. We found no recombinants among 448 progeny, suggesting that the two mutations are very close to each other and might be allelic. We tested whether several overlapping deletions for polytene chromosome region 96F–98B (which should include the mutations) caused a darkening of the \textit{P[\textit{181PRE]}\texttt{8-10C}} eye color. As none did, we suspected that both mutations are gain-of-function alleles that produce proteins with altered activities. If so, then a mutation that inactivates the mutant protein should revert the dominant suppression of the \textit{P[\textit{181PRE]}\texttt{8-10C}} eye color. Therefore, we tried to revert both mutations.
We used both X-rays and EMS to generate revertants. We recovered three X-ray-induced revertants (from 24,758 progeny) and four EMS-induced revertants (from 5500 progeny). The revertants are lethal over deficiencies for the region 96F1–98A5. By crossing to overlapping deficiencies and lethals in the region, we found that all of the revertants are lethal or semilethal mutations in the gene woc.

Sequencing of the woc gene from our original suppressor mutation chromosomes showed that both of these mutations are due to the same single amino acid change in a position evolutionarily conserved throughout the Drosophila lineage, as well as in most insects (Figure 3). This amino acid change occurs within a region of the protein with no known domain or function. We named the two original suppressor mutations wocD1 and wocD2 (for wocDominant1 and wocDominant2), and we named the revertant alleles based on the allele reverted and the mutation used (i.e., wocD1/ wocD2 was a revertant (rv) generated from wocD1 by EMS (E) mutagenesis). The wocD1/+ and wocD2/+ flies have no phenotypic defects. The wocD1 and wocD2 homozygotes survive, are fertile, and also show no phenotypic defects.

**Figure 2** Eye colors of P[181PRE]8-10C flies in wildtype and wocD1 mutants. Pictures are of eyes of 1-day-old females. All flies were w1118/ w1118, and either homozygous or heterozygous for the P[181PRE]8-10C insertion (designated by 8-10C in the figure) and wocD1 as indicated.

We also sequenced the revertants. As expected, all revertants contained the mutation present in wocD1 and wocD2, as well as an additional lesion in the woc transcription unit (Figure 3). With the exception of wocD2vX1, all of the mutants were lethal when heterozygous with all other woc mutants. The wocD2vX1, which contains a four amino acid deletion in the sixth zinc finger, is a hypomorphic allele. The wocD2vX1 survives poorly in combination with the other hypomorphic woc alleles, wocD9 and wocD6. Transheterozygous wocD2vX1/wocD9 and wocD2vX1/wocD6 flies have multiple phenotypic defects, including downturned wings, lack of wing veins, slightly rough eyes, and they are sterile.

**wocD suppresses the eye color in a position-specific manner**

P[181PRE]8-10C is inserted in the genome between the genes GstS1 and CG30456 (Figure 1). We wanted to know whether wocD modulates the PRE directly or whether it acts through regulatory DNA flanking the insertion site of P[181PRE]8-10C. Importantly, wocD does not darken the eye color of w, a mutation in the w gene that reduces the amount of w transcript and leads to orange eyes (Pirrotta and Bröckl 1984; Levis et al. 1984). This shows that wocD does not darken eye color indiscriminately. We examined whether the eye colors of flies heterozygous for other mini-white containing transgenes inserted near GstS1 were altered by wocD. We used a line with a P[EP] element inserted about 1.2 kb away from the insertion site of P[181PRE]8-10C and six lines with a P[lacW] inserted in the promoter region of GstS1 (Figure 1). The eye colors of P[lacW] or P[EP]/+; +/TM6C were compared with the eye colors of P[lacW] or P[EP]/+; wocD1/+ flies; no eye color differences were observed. This suggests that the effect of wocD on the eye color of P[181PRE]8-10C flies is dependent on the presence of the PRE in the transgene.

We next examined whether wocD could alter the eye color of flies with P[181PRE] inserted at different chromosomal locations. We used the transgene P[181PRE], which contains the same 181-prec as in P[181PRE]8-10C. In P[181PRE], the 181-bp PRE is flanked by loxP sites (see below). Because wocD dominantly alters the eye color of P[181PRE]8-10C heterozygotes, we looked at whether wocD could dominantly alter the eye color of flies heterozygous for P[181PRE] insertions that show mini-white repression. For 14 out of 15 P[181PRE] lines tested, wocD does not alter the eye color. However, in P[181PRE]8-8A, the eye color was slightly darker in a wocD mutant (data not shown). P[181PRE]8-8A is inserted just upstream of the P{G-G} regulated gene CycA (at 3L:11826614). To determine whether the effect on the eye color of P[181PRE]8-8A flies was due to the PRE, we examined the eye color of P[8]-8A flies in which the 181-bp PRE had been removed. We found that wocD had no effect on the eye color of flies that lacked the PRE. This shows that, at least at this chromosomal location, the change in eye color mediated by wocD is dependent on the PRE. However, as wocD does not influence the eye color of most P[181PRE] lines, we believe that wocD is not working on the PRE directly but on sequences flanking the P[181PRE] insertion sites.

**wocD increases the levels of GstS1 RNA in adult heads**

We examined whether the levels of GstS1 and CG30456 transcripts were altered in wocD1 mutants, both in the presence and in the absence of the P[181PRE]8-10C insertion. We examined RNA levels at three developmental stages: 3rd instar larvae, 1-day-old pupae, and adult heads. We saw no significant differences in the expression levels of GstS1 and CG30456 between wocD1 and wild-type 3rd instar larvae.
or one-day old pupae (data not shown). However, we saw a twofold increase in the level of GstS1-RNA in the adult heads of homozygous wocD1 mutants compared to wild-type (Figure 4). Flies with the P[181PRE]8-10C insert had about a twofold decrease in the expression levels of CG30456 at all developmental stages, suggesting that the insertion interferes with the transcription of CG30456 (Figure 4 and data not shown). However, wocD2 had no significant effect on the transcription level of CG30456 at any developmental stage either in wild-type or in P[181PRE]8-10C animals (Figure 4 and data not shown). We also examined GstS1 and CG30456 transcript levels in eye-antennal disks from third instar larvae of wild-type and wocD1 mutants with P[181PRE]8-10C and saw no significant differences (data not shown). Finally, we tested whether wocD altered the transcription level of CycA in adult heads. We saw no significant differences in CycA levels between wild-type and wocD1; P[181PRE] heads (data not shown).

Other dominant suppressors do not affect the expression level of GstS1 or CG30456

We tested whether four of our other dominant suppressors of pairing-sensitive silencing of P[181PRE]8-10C affect the expression levels of GstS1 or CG30456 in fly heads by qRT-PCR; we saw no effect on transcript levels of either gene (data not shown). Thus, a change in transcription level of a GstS1 is not required for suppression of the pairing-sensitive silencing of P[181PRE]8-10C. Finally, we examined the effects of three other suppressor mutations on the eye color of P[181PRE] at multiple insertion sites. Like wocD, none of the other suppressor mutations altered the eye color of P[181PRE] at the insertion sites.

A single unpaired copy of the PRE reduces the eye color of mini-white transformants

We flanked the 181-bp PRE by loxp sites and cloned it upstream of the mini-white reporter in pCaSpeR (P[L181PRE], in the same orientation and position as in the construct P[181PRE] (Figure 5). We recovered 32 lines with insertions of P[L181PRE]. Of the 25 insertion lines that were homozygous viable, 15 exhibited pairing-sensitive silencing (60%). All lines were treated with Cre recombinase to excise the 181-bp PRE, yielding P/L. None of the lines without the PRE showed pairing-sensitive silencing. In 7 of the 15 pairing-sensitive lines, the eye color of heterozygous flies became darker upon removal of the PRE, showing that some repression of mini-white expression occurred even in the heterozygotes (Figure 5). In contrast, in the lines that did not show pairing-sensitive silencing, the eye colors of flies heterozygous or homozygous for P[L181PRE] did not change after removal of the PRE, with one exception. In line P[L181PRE]11A, the eye color was slightly lighter after removal of the PRE, suggesting that this element was acting as a slight activator of mini-white expression at this location.

This result is consistent with earlier evidence that showed that the 181-bp fragment, in the context of a reporter gene driven by the en promoter, can act as either an activator or repressor of gene expression depending on the context (Devido et al. 2008). This activation activity was weak and only occurred in 1 line out of 32. This shows that, in the mini-white assay, PRE-mediated repression is the usual situation.
We determined the chromosomal insertion site for 26 of the $P[181\text{PRE}]$ lines (9 lines were lost prior to this part of the analysis) and examined whether the insertion occurred in or near a transcription unit (Table 1). We also examined whether the nearby gene is transcribed in the eye. We note that lines with pairing-sensitive silencing were just as likely to be inserted in or near genes transcribed in the eye as lines without it. Thus, insertion near a gene that is transcribed does not interfere with pairing-sensitive silencing.

**DISCUSSION**

To gain insight into the mechanism of pairing-sensitive silencing mediated by the 181-bp $en$ PRE, we conducted a genetic screen for dominant mutations that affected mini-white repression by that element. Notably, we obtained only nine mutations from screening 68,274 progeny. This low frequency of mutation recovery suggests that loss-of-function alleles were not obtained in our screen. If the loss of one copy of a gene could affect mini-white repression, these mutations would have been much more frequent. Our data suggest that mini-white repression by the 181-bp PRE is not dependent on genes that are dosage sensitive.

The classical Polycomb group mutant phenotype is the presence of sex comb teeth on the second and third legs, caused by derepression of the Sex combs reduced (Scr) HOX gene (reviewed in Kennison 1995). Scr repression is sensitive to the dose of some PcG genes, as flies with only one wild-type copy have sex comb teeth on the second and third legs. In contrast, mini-white repression via the $en$ 181-bp PRE is not sensitive to a reduction in dosage of the PcG genes (Kassis 1994 and unpublished data). The 181-bp PRE is known to bind PcG proteins, and a binding site for the DNA-binding PcG group protein Pho is required for 181-bp-mediated mini-white repression (Brown et al. 1998). Thus, we believe that PcG proteins mediate $en$ PRE mini-white repression, but that this target of PcG proteins is not dosage sensitive.

**woc$^{D1}$ and woc$^{D2}$ are gain-of-function mutations**

Heterozygosity for either $woc^{D1}$ or $woc^{D2}$ darkens the eye colors of $P[181\text{PRE}]8\text{-}10C$ flies, whereas heterozygosity of $woc$ deletions does not. This shows that $woc^{D1}$ and $woc^{D2}$ have acquired new activities and are gain-of-function alleles. Our results also show that wild-type Woc protein competes with WocD protein. This is suggested by the observation that $P[181\text{PRE}]8\text{-}10C/P[181\text{PRE}]8\text{-}10C$; $woc^{D1}woc^{-}$ flies have a darker eye color than $P[181\text{PRE}]8\text{-}10C/P[181\text{PRE}]8\text{-}10C$; $woc^{D1}$ flies. Finally, the observation that the activity of WocD is abrogated by mutations that inactivate the Woc protein shows that the woc$^{D}$ mutation alters the activity of the protein.

**A model for WocD**

The $woc$ gene encodes a zinc-finger transcription factor implicated in transcriptional activation (Wismar et al. 2000; Raffa et al. 2005) that acts, at least in part, through an association with HP1c (Font-Burgada et al. 2008; Able et al. 2009). There are five HP1 isoforms in Drosophila. Of these, HP1a is the best studied and is associated with heterochromatic DNA. In contrast, HP1c is excluded from centromeric heterochromatin and is associated with euchromatin.

As stated above woc$^{D}$ suppresses mini-white expression from $P[181\text{PRE}]$ in a position-dependent manner. Therefore, we wanted to know whether Woc binds to the genomic regions near the $P[181\text{PRE}]$ insertion sites it regulates. We were not able to obtain Woc antisera, and there is no published data showing where Woc binds in the genome. However, the Drosophila ModENCODE project has mapped the binding sites of HP1c in four different cell culture lines by chromatin immunoprecipitation followed by hybridization to tiling arrays (Kharchenko et al. 2010). Because Woc is often associated with HP1c, we examined whether there was a correlation between HP1c binding and the suppressor activity of woc$^{D}$. HP1c is bound in a cell-type–specific manner. There is no HP1c associated with the region between $GstS1$ and CG30456 in S2, Kc167, or BG3 cells; however, HP1c is bound to this region in clone 8 cells. HP1c is not associated with $CycA$ in any cell type. There is no data on HP1c localization in the pigment cells in the eye, so we cannot make any conclusion about whether WocD acts via HP1c in suppressing the PRE activity of $P[181\text{PRE}]8\text{-}10C$.

How does woc$^{D}$ affect the eye color of $P[181\text{PRE}]8\text{-}10C$? We suggest that the eye colors of $P[181\text{PRE}]8\text{-}10C$ flies result from a competition of transcriptional repression (caused by the PRE) and transcriptional activation of $GstS1$ (Figure 6). In the wild-type case, we suggest there is a competition between transcriptional activation of mini-white by flanking regulatory DNA and transcriptional repression mediated by the PRE, leading to an intermediate eye color. One prediction of this model is that if Woc levels are decreased, the PRE upstream of mini-white should be able to work more strongly, and the eye color should be lighter. Consistent with this hypothesis, the eye color of $P[181\text{PRE}]8\text{-}10C$; $woc^{D}woc^{-}$ flies is white (Figure 6). We suggest that the PRE in line $P[181\text{PRE}]8\text{-}10C$ modulates the levels of mini-white expression in part through a competition with flanking activators.

**What determines PRE activity?**

PREs have been studied for many years as silencers of homeotic gene expression in Drosophila. Recent genome-wide studies showed that PREs may play an important role in regulating gene expression levels as well. What determines whether a PRE will completely silence a gene or only decrease its expression levels? Our data suggests two things. First, the number of PREs is important. This is evident from the fact that flies homozygous for PRE-mini-white constructs, which have two
PREs, repress mini-white to a much higher level than flies heterozygous. We also note that increasing the number of PREs in cis, by duplicating a P-construct with PREs, also causes an increase in mini-white repression (Kassis 1994). Second, changes in the chromatin environment, here caused by a gain-of-function mutation in the transcriptional activator Woc, can inactivate a PRE. The dependence of PRE activity in transgenes on chromosomal environment has long been recognized and is dramatically demonstrated in a recent report (Americo et al. 2002). If the increased transcription driven by the GBS enhancer interferes with PRE activity, one would expect to see a decrease in the number of lines with pairing-sensitive silencing in this vector. However, this did not occur. Thus, increasing the transcription of mini-white itself does not alter PRE activity. In addition, we found that insertion of $P[181PRE]$ into or next to genes expressed able to obtain enough $woc^{HYP}$ adults to perform qRT-PCR, so we do not know the level of $CG30456$ and $GstS1$ RNA in these flies. This uncertainty is indicated by the question mark next to the transcription arrows in (C).

The relationship between transcriptional activation and PRE function is not simple. Addition of an enhancer containing three binding sites for the eye enhancer-activator protein Glass (GBS) to $pCaSpeR$ binding sites for the eye enhancer-activator protein Glass (GBS) to PCG proteins are designated by arrows pointing in the direction of transcription, with the height of the arrow indicating the relative level of transcription. The P-element ends (black rectangles), the 181-bp en PRE (red box), and the extent of the P[181PRE] transgene (red line at bottom) are shown. Green ovals indicate Woc activity, with WocD a bigger shape to indicate a higher activity. (Note that we have[...]

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Three lethal lines are included in this table: 8A, 10, and 21B. The eye color of line 8A heterozygous flies became lighter upon excision of the PRE; thus, we consider that this line undergoes mini-white repression by the PRE and classify it as having pairing-sensitive silencing. The eye colors of lines 10 and 21B did not change upon excision of the PRE and are classified as lines that do not show pairing-sensitive silencing. NID, no informative data.

- **Table 1** $P[181PRE]$ insertions

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<th>Line Name</th>
<th>Location</th>
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<th>Transcript Level in Eye</th>
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<td>13A</td>
<td>X:14983581</td>
<td>rab3-GEF</td>
<td>−134bp</td>
<td>173</td>
</tr>
<tr>
<td>16A</td>
<td>2L:2753118</td>
<td>CG9894</td>
<td>+319bp</td>
<td>2886</td>
</tr>
<tr>
<td>17A</td>
<td>2R:13435831</td>
<td>MESR4</td>
<td>+502bp</td>
<td>41</td>
</tr>
<tr>
<td>28A</td>
<td>2R:15556892</td>
<td>Hrg</td>
<td>−325bp</td>
<td>383</td>
</tr>
<tr>
<td>10</td>
<td>2L:3477289</td>
<td>Thor</td>
<td>−1145bp</td>
<td>1016</td>
</tr>
<tr>
<td>21B</td>
<td>2R:8475807</td>
<td>Sin3A</td>
<td>+793bp</td>
<td>NID</td>
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</tbody>
</table>
in the eye did not prevent pairing-sensitive silencing from occurring (Table 1). Therefore, we propose that it is not transcriptional activation but the actual activators present that determine whether a PRE is active or not. It has previously been suggested that PREs are general silencer elements that could act on any enhancer (Sengupta et al. 2004). The basis for this conclusion was that the Ubx PRE could act as silencers of three enhancers in reporter genes [two vestigial (vg) and one decapentaplegic (dpp) enhancer]. At that time, neither vg nor dpp was thought to be regulated by PcG proteins. However, since then, a vg PRE and a dpp PRE have been identified (Lee et al. 2005; Hauenschild et al. 2008; Okulski et al. 2011). We suggest that PREs may not be able to silence all enhancers, and in some chromosomal locations, they cannot act. It was recently reported that a human tissue–specific enhancer functions in erythroid cells by evicting PcG proteins (Vernimmen et al. 2011). Enhancers with this activity may also be present in Drosophila.

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