A Complex Array of DNA-Binding Proteins Required for Pairing-Sensitive Silencing by a Polycomb Group Response Element From the Drosophila *engrailed* Gene

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ABSTRACT

Regulatory DNA from the Drosophila gene *engrailed* causes silencing of a linked reporter gene (mini-*white*) in transgenic Drosophila. This silencing is strengthened in flies homozygous for the transgene and has been called "pairing-sensitive silencing." The pairing-sensitive silencing activities of a large fragment (2.6 kb) and a small subfragment (181 bp) were explored. Since pairing-sensitive silencing is often associated with Polycomb group response elements (PREs), we tested the activities of each of these *engrailed* fragments in a construct designed to detect PRE activity in embryos. Both fragments were found to behave as PREs in a *bxd-Ubx-lacZ* reporter construct, while the larger fragment showed additional silencing capabilities. Using the mini-*white* reporter gene, a 139-bp minimal pairing-sensitive element (PSE) was defined. DNA mobility-shift assays using Drosophila nuclear extracts suggested that there are eight protein-binding sites within this 139-bp element. Mutational analysis showed that at least five of these sites are important for pairing-sensitive silencing. One of the required sites is for the Polycomb group protein Pleiohomeotic and another is GAGAG, a sequence bound by the proteins GAGA factor and Pipsqueak. The identity of the other proteins is unknown. These data suggest a surprising degree of complexity in the DNA-binding proteins required for PSE function.

IN Drosophila, it is well documented that expression of a gene on one chromosome can be influenced by an allele of that gene on the homolog (for reviews, see Henikoff and Comai 1998; Pirrotta 1999; Wu and Morris 1999; Kennison and Southworth 2002). This phenomenon was first described at the bithorax complex by E. B. Lewis and was termed "transvection" (Lewis 1954). In that case, two alleles of *Ultrabithorax* were unexpectedly found to complement one another, and this complementation was dependent on chromosome pairing. Subsequently, many additional examples of allelic complementation have been described. At the *yellow* (*y*) gene, molecular studies have shown that enhancers located on one chromosome can activate the *y* promoter on the homolog (Geyer *et al.* 1990; Morris *et al.* 1998, 1999). This may be a common mechanism for many cases of allelic complementation. There are many other pairing-dependent effects on gene expression described in Drosophila, and these may occur by different mechanisms. For example, a particular allele of the brown locus, bw^D, is able to inactivate a paired wild-type allele of *bw*. This silencing is thought to be due to the ability of heterochromatic sequences present at bw^D to bring the wild-type copy into an inactive part of the nucleus (Csink and Henikoff 1996; Dernburg *et al.* 1996).

Pairing-dependent silencing has also been seen for transgenes in Drosophila. The commonly used *P*-element transformation vector *CaSpeR* includes a minigene for the selectable marker *white*; expression of the *white* minigene causes *white* mutant (white-eyed) flies to have colored eyes (Pirrotta 1988). Eye color is sensitive to the dosage of *white*, with higher levels of *white* mRNA leading to darker eye colors. Thus, flies homozygous for a *CaSpeR* transposon, having two copies of mini-*white*, have a darker eye color than heterozygotes. However, when particular fragments of regulatory DNA are included in *pCaSpeR*, the eye color of homozygotes is lighter than that of heterozygotes (reviewed in Kassis 2002). This phenomenon has been called "pairing-sensitive silencing" (Kassis 1994). DNA fragments that cause pairing-sensitive silencing are called pairing-sensitive elements (PSEs). The focus of our laboratory is to understand the molecular mechanisms and biological significance of pairing-sensitive silencing.

Pairing-sensitive silencing was first described for DNA sequences from the Drosophila *engrailed* gene and subse-

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quently found for many other DNA fragments (Kassis *et al.* 1991; Fauvarque and Dura 1993; Chan *et al.* 1994; Gindhardt and Kaufman 1995; Kapoun and Kaufman 1995; Hagstrom *et al.* 1997; Fujioka *et al.* 1999; Muller *et al.* 1999; Shimell *et al.* 2000). Many of these DNA fragments are known to be regulatory elements for the Polycomb group genes [Polycomb group response elements (PREs); Fauvarque and Dura 1993; Simon *et al.* 1993; Chan *et al.* 1994; Gindhardt and Kaufman 1995; Hagstrom *et al.* 1997; Muller *et al.* 1999; reviewed in Pirrotta 1997a,b]. The Polycomb group (PcG) genes are a diverse group of at least 15 genes, important for heritable gene silencing in Drosophila (reviewed in Bienz and Müller 1995; Kennison 1995; Hagstrom and Schedl 1997; Pirrotta 1997a,b). Many PcG genes encode chromatin-associated proteins that are components of large protein complexes (Satijn and Otte 1999; Shao *et al.* 1999; Ng *et al.* 2000; Chang *et al.* 2001; Saurin *et al.* 2001; Tie *et al.* 2001). The PcG proteins act in three or more distinct protein complexes that silence transcription by unknown mechanisms. These mechanisms may involve alterations in chromatin structure and modification of histones (reviewed in Satijn and Otte 1999; Francis and Kingston 2001; Mahmoudi and Verrijzer 2001). Pairing-sensitive silencing may be caused by the actions of PcG proteins; however, the relationship is not simple. PREs seem to be composite elements that can be divided into many PSEs, and single PSEs often cannot work as PREs (Horard *et al.* 2000; Shimell *et al.* 2000). Thus, a PSE may be a component of a PRE, but in general is not sufficient for PRE activity. The molecular mechanisms that account for PSE activity are far from clear.

We have been studying the sequences important for the pairing-sensitive silencing activity of a PSE from the Drosophila *engrailed* gene. We have previously shown that the protein encoded by the PcG gene *pleiohomeotic* (*pho*) binds to sequences within an *engrailed* PSE and that those sequences are required for PSE activity (Brown *et al.* 1998). In this report we further examine the sequences important for PSE activity by mutational analysis of a minimal PSE and by gel mobility shift assays, using nuclear extracts from Drosophila embryos to detect protein-binding sites. Our data show that at least five and perhaps as many as eight distinct protein-binding sites are required for full PSE activity. We compared the pairing-sensitive silencing activity of a 181-bp PSE with a larger *engrailed* fragment (2.6 kb) that contains both this PSE and another strong PSE and found that they behave similarly in most assays, although silencing by the larger fragment was more stable in one assay. Finally, we compared the PRE activities of the 2.6-kb fragment and the 181-bp fragment in embryos in the context of a *bxd-Ubx-lacZ* reporter transgene. We found that each of these fragments could act as a PRE, while the larger fragment contained additional silencing capabilities.

MATERIALS AND METHODS

Construction of transgenes: The *en* fragment in *2.6up*, *2.6upR*, *2.6D*, and *2.6DR* extends from −2.407 kb to −10 bp, just upstream of the transcription start site. A natural *Eco*RI site is present at the 5′ end, and a synthetic *Eco*RI site was added to the 3′ primer. The 3′ primer differed from the natural sequence at two positions: at −20 bp, a G was present instead of a C, and at −21 bp, a C was present instead of a G. These changes are not expected to change pairing-sensitive silencing (PSS) activity since this activity has been mapped to sequences upstream of −400 bp (Kassis *et al.* 1991). The *en* fragment in *181up*, *181upR*, and *181D* extends from −576 to −395 bp. This is the same fragment used in Kassis (1994, construct 8) and in Brown *et al.* (1998, Figure 1). We previously stated that this fragment ended at −400 bp, at the beginning of a *Sph*I site; however, this was incorrect, since the entire *Sph*I site was present. Therefore, the correct 3′ end is −395 bp. Synthetic *Eco*RI sites were added to the 5′ and 3′ primers used to amplify the 181-bp fragment. For cloning the *en* fragments upstream of the mini-*white* gene, *pCaSpeR* was cut with *Eco*RI, and the *en* fragments were cloned as *Eco*RI fragments. The orientations of the *en* fragments were determined by PCR. For cloning the *en* fragments downstream, *pCaSpeW15* was cut with *Sst*I, removing a fragment of the mini-*white* gene including an upstream *Eco*RI site, creating *pCaSpeW15-Sst*I. The *en* fragments were then cloned as *Eco*RI fragments into *Eco*RI-cut *pCaSpeW15-Sst*I, and the orientation of insertion was determined by PCR. The *Sst*I fragment was then cloned back into the constructs.

For *Δ1*, *Δ2*, *Δ4*, and *Mutsite1*, the 181-bp fragment was amplified by PCR and cloned into M13mp19. The conserved sites were deleted or mutated using the Bio-Rad (Richmond, CA) Mutagene M13 *in vitro* mutagenesis kit. Mutagenized clones were identified either by DNA sequencing (for the deletion clones) or by the identification of clones with an additional *Rsa*I site (for the mutations), followed by DNA sequencing. The mutagenized fragments were amplified by PCR and subcloned into *pCaSpeR* (Pirrotta 1988). All clones were sequenced prior to injection.

For constructs *Δ5*, *12*, *13*, *14*, *16/4*, *139 bp*, and *98 bp*, PCR primers were designed to amplify the following *en* fragments: Δ5, −576 to −412; 12, −454 to −395; 13, −516 to −437; 14, −576 to −499; 16/4, −516 to −395; 139, −576 to −437; and 98, −550 to −452. The 5′ primer always had a synthetic *Eco*RI site added, and the 3′ primer always had a synthetic *Bam*HI site added. The *en* fragments were cloned into *Bam*HI/*Eco*RI cut *CaSpeR*. All constructs were then sequenced. The results from construct *12* were previously reported (Kassis 1994). The 139-bp fragment was also cloned into a vector with three binding sites for the eye enhancer binding protein Glass (Fujioka *et al.* 1999).

For *Mutsite2*, *MutGAGA*, *MutNC1*, and *MutNC2*, mutations were made using mutated primers in the PCR reactions. The 5′ primer started at −576 bp and the 3′ primer started at −437 bp. For the synthetic constructs, the oligonucleotides (oligos) used are listed in Figure 5. Oligos for both strands were made, annealed, and cloned into *pCaSpeR*. For *Synsites3*, the annealed oligos were ligated in the presence of *Bgl*II prior to cloning into the *Bam*HI site of *pCaSpeR*. The sequences of these clones were determined.

For the *en-bxd-Ubx* constructs, the vector *Uβglz* containing 3.1 kb of the *Ubx* promoter fused to the *lacZ* gene was cut with *Xba*I-*Kpn*I, and a 1.6-kb *Xba*I-*Kpn*I fragment containing the BXD enhancer was inserted (vectors obtained from J. Müller; Müller and Bienz 1991). The *en* fragments extending from −2.407 kb to −10 bp (*2.6en*) and from −537 to −395 bp (*181en*) were amplified using primers containing synthetic

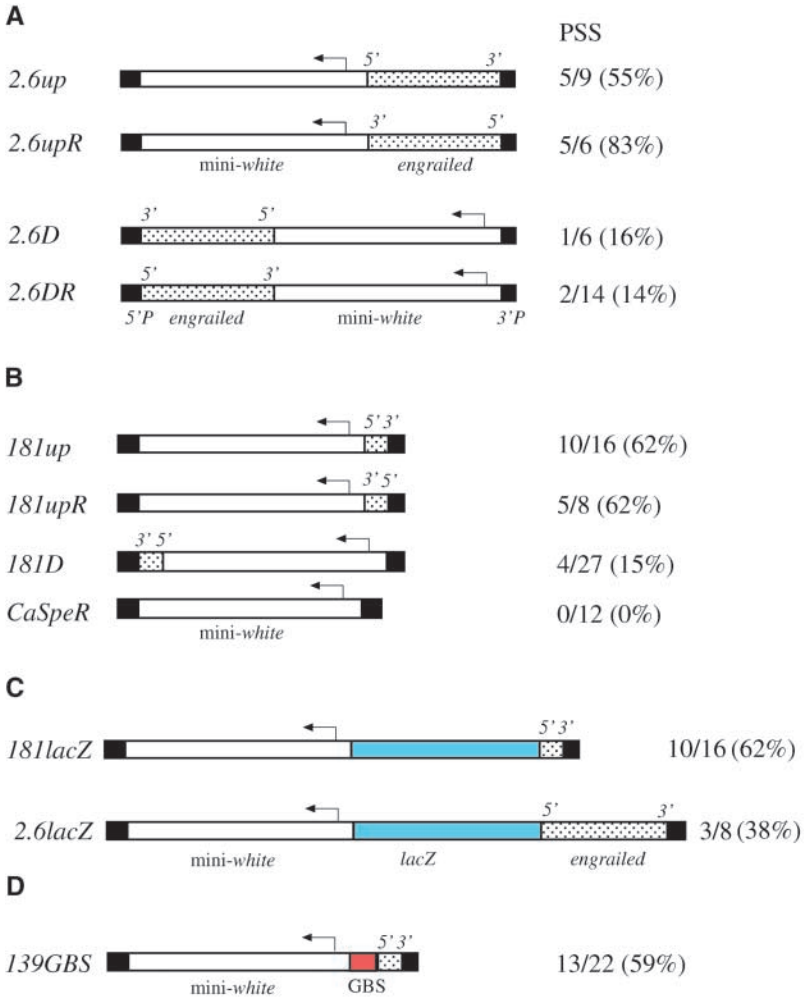

FIGURE 1.—Pairing-sensitive silencing of mini-*white* by *engrailed* DNA. Construct names are on the left, followed by a diagram of each construct and the number of lines showing PSS, over the total number of homozygous viable lines. The corresponding percentage of lines showing PSS is in parentheses. Black boxes represent *P*-element ends, open boxes are the mini-*white* gene, and stippled boxes are *engrailed* DNA. Constructs are approximately to scale. 5′ and 3′ represent the orientation of the *engrailed* DNA with respect to its promoter. Arrows indicate the start site and direction of mini-*white* transcription. (A) Constructs with the 2.6-kb *engrailed* fragment cloned upstream and downstream in both orientations. (B) Constructs with the 181-bp *engrailed* fragment cloned upstream in both orientations and in one orientation downstream. The starting construct *CaSpeR* is also shown to illustrate that the small percentage of lines showing PSS with the *engrailed* DNA cloned downstream of mini-*white* is greater than that obtained with *CaSpeR* alone. (C) The *E. coli lacZ* gene (blue box) was cloned between the mini-*white* promoter and *engrailed* DNA. (D) Three binding sites for the eye disc transcriptional activator protein Glass (GBS, red box) were cloned between the mini-*white* promoter and the *engrailed* fragment.

*Xba*I sites. The orientation of the *en* fragments in this vector was 3′ to 5′ with respect to the *Ubx* promoter.

Generation and analysis of transgenic lines: Injections were done into homozygous *Df(1)w67c2, y* embryos using procedures described in FUJIOKA *et al.* (2000). Most of the lines described in this study were obtained directly from injection, but a few were generated by transposon mobilization using a *P[Δ2,3], 99B* line (ROBERTSON *et al.* 1988). The eye color of homozygotes and heterozygotes was compared in flies of the same age and sex. For this report, lines were scored as having PSS only if the eye color of homozygotes was lighter than that of heterozygotes. In previous studies (KASSIS *et al.* 1991; KASSIS 1994), lines were also scored as having PSS if the eye color of homozygotes was the same as that of heterozygotes. We previously reasoned that the eye color of homozygotes should be darker, and thus, when it was not, PSS was occurring. However, in recent experiments we have observed that lines generated with either *pCaSpeR* alone or with *pCaSpeR* containing non-PSS DNA fragments yield flies with the same eye color in homozygotes and heterozygotes at a frequency of 5–10%. Therefore, in this report, lines with the same eye color in homo- and heterozygotes were scored as negative for PSS activity.

Gel mobility shift assays: Nuclear extracts were made from 0- to 22-hr Drosophila embryos as described in TSUKIYAMA and WU (1995). Three microliters of nuclear extract was incubated in a total volume of 10 μl with 20 fmol labeled oligo, 10 μg tRNA, 4 μg poly(dI-dC), 100 mM KCl, 35 mM HEPES pH 7.9, 1 mM dithiothreitol, 50 μM $ZnCl_2$, 12% glycerol, 2 mM spermidine, and 5 mg/ml BSA. Samples were incubated for 20 min at 25°, supplemented with 2 μl 5% BSA and 2 μl gel-loading buffer (2.5% Ficoll 400, 0.5× TBE and tracking dyes), and then electrophoresed on a 1% agarose, 0.5× TBE gel. The gel was dried onto DE81 paper (Whatman) and autoradiographed.

RESULTS AND DISCUSSION

We previously showed that a 2.6-kb fragment containing the *engrailed* promoter and 2.4 kb of upstream sequences mediated pairing-sensitive silencing of the mini-*white* gene in *CaSpeR*, at ~60% of insertion sites (KASSIS *et al.* 1991). Here we tested the orientation dependence of that activity. We found that this fragment works equally well in either orientation when positioned upstream of the mini-*white* transcription unit, but that the activity was significantly reduced when placed downstream, in either orientation (Figure 1A). We also tested the activity of a 181-bp subfragment (extending from −576 to −395 bp, construct 8 in KASSIS 1994) in both orientations upstream and in one orientation downstream of the mini-*white* gene (Figure 1B). Like the

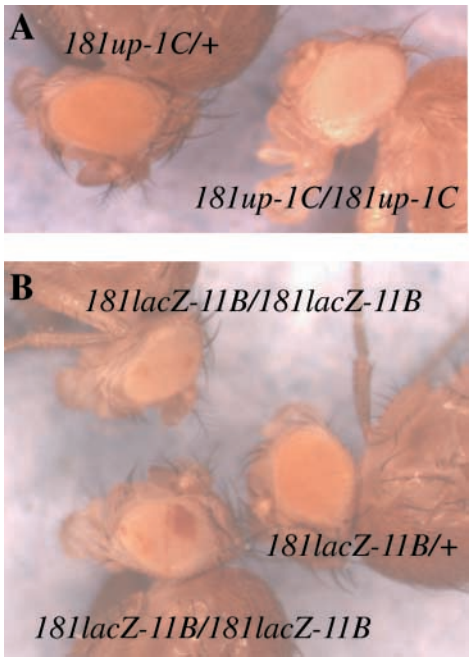

FIGURE 2.—Pairing-sensitive silencing by the 181-bp PSE. (A) The eye color of flies from line *181up-1C* indicates that the mini-*white* gene is homogenously silenced in homozygotes. (B) The mini-*white* gene in line *181lacZ-11B*, where the *engrailed* PSE is 3.9-kb upstream of the promoter, is silenced in a variegated manner. Homozygotes contain patches of red on a yellow background, and the distribution and extent of eye coloration differ from eye to eye.

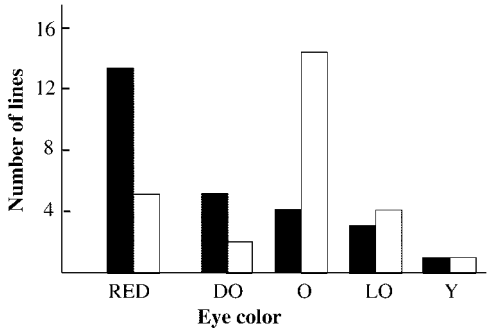

FIGURE 3.—Glass binding sites darken the eye color of heterozygous flies. The number of lines scored is shown on the *y*-axis and the eye colors observed (RED, red; DO, dark orange; O, orange; LO, light orange; Y, yellow) are shown on the *x*-axis. Solid bars represent *139GBS* heterozygotes; open bars represent heterozygotes with construct *139bp*.

larger fragment, there was no difference in the percentage of lines showing pairing-sensitive silencing when the 181-bp fragment was tested in either orientation upstream. However, the pairing-sensitive silencing activity was greatly reduced when cloned downstream of mini-*white*. To test if this decreased activity was the result of an increased distance between the mini-*white* promoter and the PSE, we inserted the *lacZ* gene of *Escherichia coli* between the mini-*white* promoter and the *engrailed* DNA (Figure 1C). This separated the *engrailed* fragments from the mini-*white* promoter by 3.9 kb, similar to the 3.7-kb distance between them when the *engrailed* fragments were downstream of mini-*white*. The increased distance had no significant effect on the percentage of lines showing pairing-sensitive silencing (Figure 1C). Therefore we conclude that the *engrailed* PSEs can function at a considerable distance upstream of the mini-*white* promoter.

Interestingly, pairing-sensitive silencing from the upstream position by the 2.6-kb *engrailed* fragment was more stable than that by the 181-bp fragment, in that the *2.6lacZ* construct resulted in eyes of a homogeneously lighter eye color, similar to that seen when either the 181-bp or the 2.6-kb fragment was located adjacent to the mini-*white* promoter (Figure 2A). In contrast, with *181lacZ* in the upstream position, the repression was not homogenous. Instead, the eye color of homozygotes was often white or yellow with red spots (Figure 2B), suggesting that while the transgene was silenced in some portions of the eye, in others both copies were expressed, resulting in the darker eye color. The size and location of the spots varied from eye to eye, suggesting that loss of repression could occur either early or late in development and that the unrepressed state was thereafter heritably transmitted through multiple cell divisions. Of the 10 *181lacZ* lines that gave pairing-sensitive silencing, 9 had such variegated eyes in homozygotes. In contrast, of the three *2.6lacZ* lines that gave pairing-sensitive silencing, none had variegated eyes. Thus, the stability of pairing-sensitive silencing through development and cell division is a distinct property of PSEs independent of the strength of repression that they produce within single cells.

We wondered why pairing-sensitive silencing occurs at only 50–60% of chromosomal insertion sites. Studies on pairing-sensitive silencing by the *bxd* PRE have shown that this PRE can completely silence mini-*white* at some chromosomal positions even in heterozygotes (SIGRIST and PIRROTTA 1997). If the *engrailed* fragment behaved in a similar manner, then there would exist a class of transgenic lines with white eyes, which would not be recovered, and this would result in the percentage of pairing-sensitive lines recovered being artificially low. We therefore tested the minimal 139-bp PSE (defined below) in a vector that contained three binding sites for the eye enhancer-activator protein Glass (ELLIS *et al.* 1993). We reasoned that the eye color of lines recovered should be darker using this glass-binding site (GBS) vector and that we might recover a greater percentage of lines with pairing-sensitive silencing. As expected, the eye color of heterozygous flies was on average darker with the GBS vector (Figure 3). However, the percentage of lines showing pairing-sensitive silencing was the same with both vectors (Figure 1D and Figure 4B, *139bp* construct). Thus, the 139-bp PSE does not appear to be a strong silencer in the heterozygous state. These data

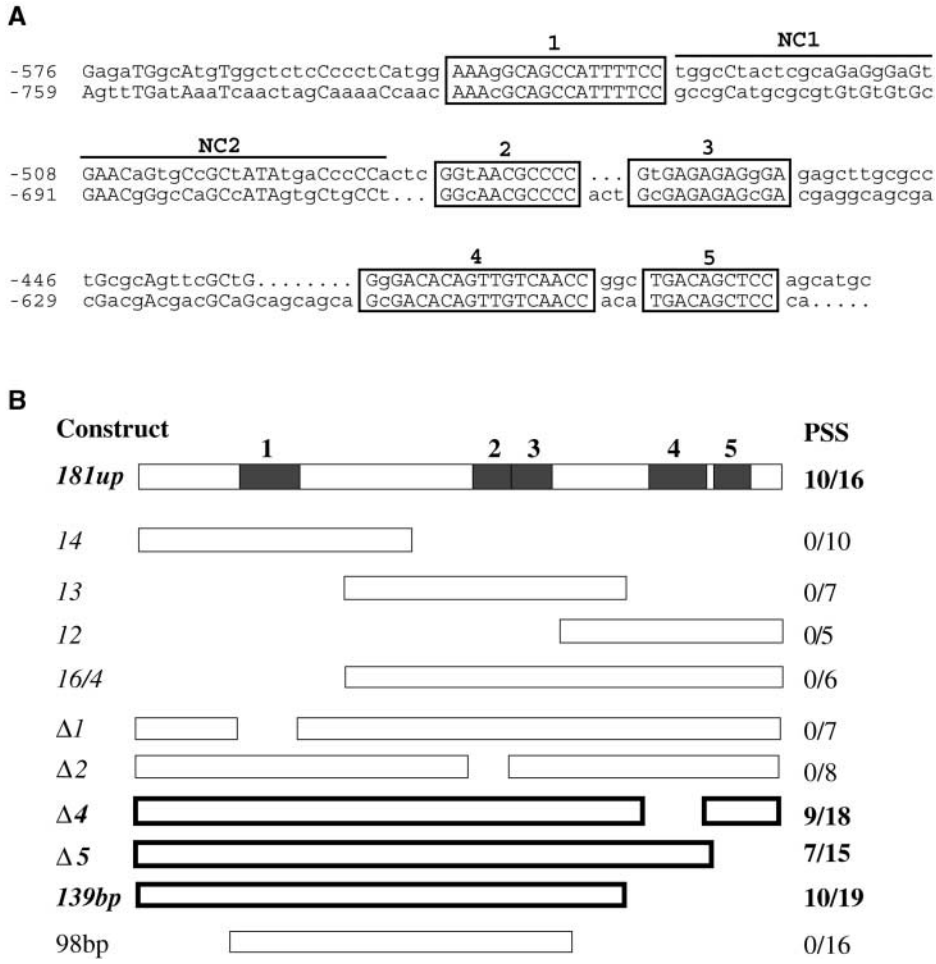

FIGURE 4.—Defining a minimal PSE. (A) A sequence comparison of *D. melanogaster* (top sequence) and *D. virilis* (bottom sequence) is shown (from KASSIS *et al.* 1989). Identical bases are capitalized. Nonidentical sequences are shown in lowercase letters. Dots indicate a gap put in the sequence to optimize alignment. Long stretches of sequence identity are boxed and labeled 1–5. The two nonconserved regions mutated in Figure 6 are shown by lines above and denoted NC1 and NC2. (B) The starting construct *181up* is shown on the top line along with the position of the conserved sequence blocks (stippled and numbered boxes). The DNA present in each construct is shown by a rectangle(s). Δ*1*, Δ*2*, and Δ*4* are all precise internal deletions of the conserved bases shown boxed in A. The number of lines that showed PSS over the total number of homozygous viable lines is shown on the right. Those constructs that were positive for PSS are shown in boldface type. The data from Δ*1* have previously been reported (BROWN *et al.* 1998).

also show that the 139-bp PSE is able to silence a strong eye enhancer.

Identification of sequences important for pairing-sensitive silencing: Our primary goal is to understand the molecular mechanisms responsible for pairing-sensitive silencing. To begin to accomplish this, we attempted to identify all the DNA-binding proteins important for the activity of a single PSE. Our first goal was to identify a minimal PSE. We started with the 181-bp fragment that we had previously identified as sufficient for pairing-sensitive silencing activity (KASSIS 1994). We used sequence conservation with a distantly related Drosophila species, *Drosophila virilis*, to guide our analysis (estimated divergence time 60 million years; BEVERLEY and WILSON 1984). We previously showed that the homologous DNA fragment from *D. virilis* acts as a PSE in *D. melanogaster* (KASSIS 1994). A sequence comparison of these two fragments is shown in Figure 4A (sequence data from KASSIS *et al.* 1989). Long blocks of high sequence similarity are shown in boxes and are labeled 1–5. Other sequence identities are shown in capital letters. Two regions with no long stretches of sequence identity are overlined and labeled NC1 and NC2 (for nonconserved regions 1 and 2). Conserved box 1 contains the binding site for the PcG protein Pho (BROWN *et al.* 1998). Conserved box 3 contains the sequence GAGAG, a binding site for GAGA factor and Pipsqueak, two proteins thought to be important in PRE function (SOELLER *et al.* 1993; LEHMANN *et al.* 1998; HORARD *et al.* 2000; BUSTURIA *et al.* 2001; HODGSON *et al.* 2001). In gel mobility band shift experiments to detect DNA-binding proteins, we found evidence for protein binding to sequences present in conserved box 2, but not to sequences present in conserved boxes 4 and 5 (below and data not shown). We began our analysis by subdividing the 181-bp fragment and by deleting separately the sequences present in boxes 1, 2, 4, and 5. In Figure 4B, rectangular boxes represent the sequences present in each construct. The positions of the conserved sequences are indicated by stippling in the *181up* diagram. Those constructs that retain pairing-sensitive silencing activity are shown in boldface type. The smallest active construct is *139bp*, which contains conserved sequences 1–3. Thus, conserved regions 4 and 5 are not required for pairing-sensitive silencing.

We next made synthetic constructs using sequences present in boxes 1–3 and asked whether they were sufficient to function as PSEs. Three copies of the Pho-binding site (*3X Pho*, Figure 5) had no pairing-sensitive silencing activity. We also tested a synthetic construct containing a Pho site, site 2, and site 3 (GAGAG) as

CONSTRUCT	SEQUENCE	PSS
3XPho	GGATCC (AAAGGCAGCCATTTTCC)$_3$ AGATCC *BamHI* Pho site *BglII/BamHI*	0/8
SynSites	GAATTC CAGCCATTTT GGTAACGCCCC GTGAGAGA GGATCC *EcoRI* Pho site Site 2 GAGA/Psq *BamHI*	1/23
SynSites3	GGATCC (CAGCCATTTT GGTAACGCCCC GTGAGAGA AGATCC)$_3$ *BamHI* Pho site Site 2 GAGA/Psq *BglII/BamHI*	1/11

FIGURE 5.—PSE activities of synthetic constructs. The name of each construct is shown on the left, followed by the sequence. On the right, the number of lines with PSS is shown over the total number of homozygous viable lines.

either one or three copies (Figure 5). With one copy, 1/23 lines showed pairing-sensitive silencing, and with three copies, 1/11 lines had this activity. While these numbers are very low and not significantly different from 0/23 or 0/11, we believe that the synthetic construct has a very low level of PSE activity because pairing-sensitive silencing is observed very infrequently or not at all with CaSpeR alone (Figure 1 and our unpublished results). However, it is clear that some of the sequences required for pairing-sensitive silencing were not present in these synthetic constructs.

We tested whether base substitutions that disrupt the Pho and GAGAG binding sites compromised the activity of the PSE. Both of these changes decreased the percentage of lines showing pairing-sensitive silencing to a very low level (*MutSite1* and *MutGAGA*, Figure 6). Likewise, mutations in conserved site 2 similarly reduced the PSE activity (*MutSite2*, Figure 6). As a control, we introduced two groups of mutations into the less conserved region between sites 1 and 2. To our surprise, the 6-nucleotide substitution in the region labeled NC1 in Figure 4A greatly reduced pairing-sensitive silencing activity (*MutNC1*, Figure 6). Likewise, the 10-nucleotide substitution in NC2 disrupted the pairing-sensitive silencing activity of the 139-bp PSE (*MutNC2*, Figure 6). With these data in hand, we investigated whether proteins in nuclear extracts from Drosophila embryos could recognize these sequences *in vitro*.

CONSTRUCT	MUTATIONS	PSS
MutSite1	TACG AAAgGCAGCCATTTTCC	1/11
MutSite2	GTACG GGtAACGCCCC	1/11
MutGAGA	C G C T c GtGAGAGAGgGAgag	2/16
MutNC1	T g t C c T tggcCtactcgcaGaGgGaGt	2/19
MutNC2	T T t TA TA c g G GAACaGtgCcGCtATatgaCccCC	0/14

FIGURE 6.—Effects of base pair substitutions on pairing-sensitive silencing. The name of each construct is shown on the left, followed by the wild-type sequence. The mutations introduced are shown above each wild-type sequence. The substitutions in *MutSite1* were made in the 181-bp PSE, and the results were previously reported (BROWN *et al.* 1998). All other mutations were made in the 139-bp PSE. The number of lines with PSS is shown over the total number of homozygous viable lines.

Eight protein-binding sites in the 139-bp PSE: We previously showed that conserved site 1 binds the PcG protein Pho (BROWN *et al.* 1998). Further sequence analysis identified an additional Pho site on the opposite strand, from −569 to −576: GCCATCTC, which matches the Pho consensus (G/t)CCATN(T/a)(T/g/c) (HYDE-DERUYSCHER *et al.* 1995). As previously stated, conserved site 3 contains the sequence GAGAG, a binding site for both GAGA factor and Pipsqueak (SOELLER *et al.* 1993; LEHMANN *et al.* 1998). An additional GAGAG sequence is present on the opposite strand and extends from −557 to −562. Thus, two Pho-binding sites and two GAGAG sequences are present in the 139-bp PSE. Within the 98-bp construct that does not have PSE activity, one Pho and one GAGAG site have been deleted. In the homologous *D. virilis* DNA fragment, the only GAGAG sequences are those present in conserved site 3; however, two Pho sites are present, one within conserved site 1 and one located in the nonconserved region (within NC2, GCCATAGT). Thus it is possible that two Pho sites may be required for PSE function. This has not yet been tested.

We examined whether conserved site 2 could bind a factor present in nuclear extracts from Drosophila embryos. Oligos containing the site 2 sequence were radioactively labeled and combined with nuclear extracts from Drosophila embryos in gel mobility shift assays. Since an 11-bp oligo containing only site 2 did not give a reproducible band shift, we used a 19-bp oligo for our experiments. Results from a representative gel shift experiment are shown in Figure 7A. In this experiment, one band was detected that was effectively competed with an unlabeled site 2 19-mer, but not by an oligo containing the 5-bp mutation used to test the function of conserved site 2 *in vivo* (Figure 6). Our data suggest that at least one protein binds conserved site 2 and that mutation of this site leads to a loss of pairing-sensitive silencing activity.

We next examined whether we could detect proteins binding to NC1 and NC2. We found that at least three proteins can bind within this region. The sequences of the NC1 and NC2 regions and the oligos used in gel

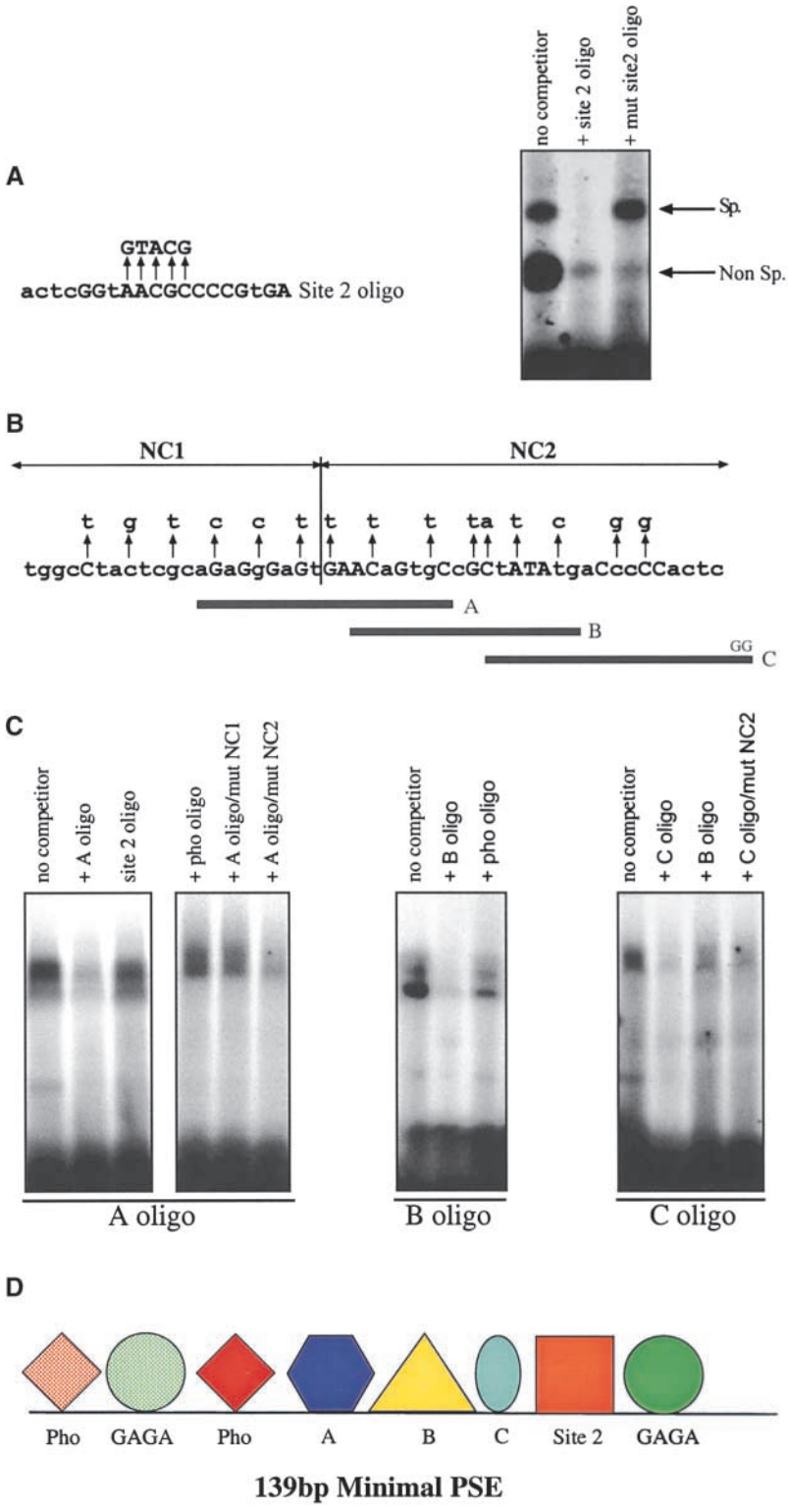

FIGURE 7.—Gel mobility shift assays. (A) The site 2 oligo (sequence shown) was radioactively labeled and used in a gel shift experiment with Drosophila embryonic nuclear extracts. A single specific complex was detected that was competed by a 100-fold excess of the site 2 oligo, but not by a site 2 oligo with 5 nucleotide substitutions (MutSite2 oligo). Nucleotide substitutions are shown above the wild-type sequence. The band labeled nonspecific (Non Sp.) can be competed by any oligonucleotide (data not shown). (B) The sequence of the two nonconserved regions, NC1 and NC2, is shown, with the nucleotide substitutions in mutated oligos shown above the wild-type sequence. The oligos used in the band-shift experiments in C are shown by lines labeled A–C below the sequence. The C oligo extends beyond NC2 by 2 bases, both G's. (C) Gel mobility shift assays using the A, B, and C oligos. (Left) Mobility shift of the A oligo in the absence of competitor DNA or in the presence of a 100-fold excess of unlabeled A oligo, site 2 oligo, Pho oligo, or the A oligo with the NC1 or the NC2 mutations. (Middle) B oligo mobility shift in the absence of competitor DNA or in the presence of a 100-fold excess of either unlabeled B or Pho oligo. (Right) C oligo mobility shift in the absence of competitor DNA or in the presence of a 100-fold excess of unlabeled C oligo, B oligo, or the C oligo with the NC2 mutations. (D) Summary of the factors that bind to the 139-bp minimal PSE. The stippled Pho and GAGA icons represent sites that show homology to the consensus-binding sites for these factors but that we have not tested for binding.

shift experiments are shown in Figure 7B. We did not find evidence of a protein binding to the NC1 sequence alone. However, using a 19-bp oligo spanning the overlap between NC1 and NC2 (oligo A), we detected a reproducible band shift, suggesting that a protein binds to this region. The sequence of this region bears some similarity to a GAGA-element binding sequence (the sequence is GAGGGAG) but was not competed by a GAGAGAG oligo (data not shown). The band shift with the A oligo was specifically competed by itself (A oligo), but not by the site 2 or Pho oligo or by the A oligo with the mutations present in NC1. Thus this factor should not bind to the PSE present in lines carrying the *MutNC1* construct (which showed very low level activity; Figure

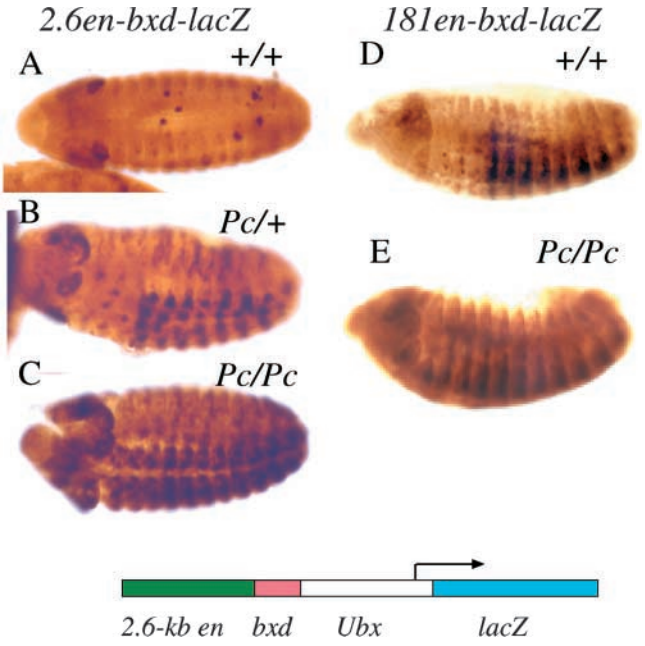

FIGURE 8.—PRE activities of *engrailed* DNA. The DNA construct used to test for PRE activity is diagrammed at the bottom. One construct contained 2.6 kb of *engrailed* DNA in the position shown (*2.6en-bxd-lacZ*), while the other contained the 181-bp *engrailed* PSE in the same position (*181en-bxd-lacZ*, not diagrammed). β-Galactosidase expression was visualized by immunoperoxidase staining. Embryos are anterior left, ventral (A), ventral-lateral (B and C), and lateral views (D and E), at ~10 hr after egg laying. (A–C) *2.6en-bxd-lacZ* in wild type (A), a *Pc* heterozygote (B), or a *Pc* homozygote (C). (D and E) *181en-bxd-lacZ* in wild type (D) or a *Pc* homozygote (E).

6). In contrast, an A oligo carrying the NC2 mutations could still compete with the A oligo band shift. Therefore, binding of the A factor should be unaffected in the lines carrying the NC2 mutations. When the NC2 oligo was used in a band shift experiment, multiple specific bands were observed (not shown). We therefore divided it into two different oligos, B and C (Figure 7B). The B oligo has substantial overlap with the A oligo, but they did not cross-compete for shifted complexes (data not shown). Three specific bands were detected in a mobility gel shift experiment using the B oligo (Figure 7C). The mutated B oligo, carrying the NC2 mutations, partially competed with the unmutated B oligo (not shown), suggesting that the binding of factor B may be decreased in the *MutNC2* construct. For the C oligo, a specific complex was detected, and this complex was not competed by either a B oligo or a C oligo carrying the same mutations as in the *MutNC2* construct. Thus, the binding of both factors B and C is disrupted in the PSE carrying the NC2 mutation (*MutNC2*, Figure 6). Therefore, while we can surmise that the binding of at least one factor to the NC2 region is important for PSE function, we cannot state whether it is factor B or C or both. Taken together, these data indicate that there are eight protein-binding sites in the 139-bp PSE (Figure 7D). Our functional data show that the conserved Pho site, site A, site B or C (or both), site 2, and the conserved GAGAG sites are all important for PSE activity (Figure 6). Mutations in the nonconserved Pho and GAGAG sites have not yet been tested.

PRE activity of the *engrailed* fragments: PREs often act as PSEs in *CaSpeR* vectors, and there are several reasons to suggest that the *engrailed* DNA we are studying is a PRE. First, the fact that it acts as a PSE suggests that it might be a component of a PRE. Second, Pho, a PcG protein, is required for pairing-sensitive silencing activity (BROWN *et al.* 1998). Third, STRUTT and PARO (1997) found that the PcG proteins Polycomb, Posterior sex combs (Psc), and Polyhomeotic could be cross-linked to sequences within the 2.6-kb *engrailed* fragment in cultured cells, while, of the proteins tested, only the Psc protein could be cross-linked to the 181-bp PSE. We tested whether *engrailed* PSEs could act as PREs in a vector designed to test for PRE activity in embryos. This vector includes a 3.1-kb *Ubx* promoter fused to *lacZ* and the 1.6-kb *bxd* enhancer (MÜLLER and BIENZ 1991). Without a PRE, *lacZ* is expressed throughout both the ectoderm and the nervous system in late-stage embryos, while addition of a PRE restricts *lacZ* expression to PS6 and posterior segments (MÜLLER and BIENZ 1991). This vector was used to study the *iab*-7 PRE (HAGSTROM *et al.* 1997). Interestingly, when the 2.6-kb *engrailed* fragment was tested in this vector, it largely silenced the activity of the *bxd* enhancer in a wild-type background. In the embryo shown in Figure 8A, *lacZ* was expressed in a few cells in the central nervous system, in very light stripes in the ectoderm (due to the "basal activity" of *lacZ* vectors), and in the head (due to regulatory sequences flanking the position of insertion). Twelve lines of *en2.6-bxd-lacZ* were recovered. Six had expression patterns that were due to the position of insertion and could not be interpreted. Three of the lines had very limited *lacZ* expression in the nervous system and no expression in the ectoderm, similar to the embryo shown in Figure 8A. Individual embryos varied as to the number and position of cells expressing *lacZ*, although expressing cells were always posterior to PS5. Thus, the decision of whether or not to silence was made on a cell-by-cell basis. In a heterozygous *Pc* background, some of this silencing was relieved, and *lacZ* was expressed more extensively in the nervous system. However, this expression was still restricted to PS6 and posteriorly (Figure 8B). Three *en2.6-bxd-lacZ* lines gave embryos with expression patterns similar to that seen in Figure 8B in a wild-type background. In *Pc* homozygotes, *lacZ* expression was derepressed anterior to PS6 in all six lines, while remaining restricted primarily to the nervous system. Although the *bxd* enhancer is also active in the embryonic ectoderm, *lacZ* was expressed only lightly in the ectoderm in all six *en2.6-bxd-lacZ* lines, even in *Pc* homozygotes. Thus, sequences within the 2.6-kb fragment were able to partially silence the *bxd* enhancer in the

ectoderm in a *Pc*-independent manner. We suggest that these may be *engrailed* regulatory sequences that normally act to keep *engrailed* off in the anterior compartment of each segment. Since the 2.6-kb *engrailed* fragment does silence the *Ubx* promoter in a Polycomb-responsive manner in the nervous system, it is behaving as a PRE in that tissue. We also examined whether *lacZ* expression in these lines differed in embryos heterozygous or homozygous for the reporter construct. We saw no evidence for increased silencing in homozygotes, suggesting that the silencing activity is not pairing sensitive in embryos (data not shown).

In contrast to the 2.6-kb fragment, the 181-bp PSE behaves as a typical PRE in the *bxd-Ubx-lacZ* reporter. In wild-type embryos, *lacZ* was expressed in both the ectoderm and the nervous system in PS6 and posteriorly (Figure 8D). In *Pc* homozygotes, *lacZ* expression extended anteriorly (Figure 8E). There was no derepression in *Pc* heterozygotes (data not shown). For *181en-bxd-Ubx-lacZ* we obtained 12 lines. Two had enhancer trap patterns, and of the remaining 10, 8 showed expression restricted to PS6 and posteriorly. The degree of restricted expression varied with the insertion site, with some lines showing very good anterior silencing and others having many individual *lacZ*-expressing cells anterior to PS6.

CONCLUDING REMARKS

Here we present a functional analysis of the sequences required for pairing-sensitive silencing and of the protein-binding capabilities of a 139-bp minimal PSE. This analysis suggests that binding sites for at least five different DNA-binding proteins are required for full activity of this PSE, with some sites still untested. One of the essential sites binds Pho, the product of the Polycomb group gene *pleiohomeotic* (Brown *et al.* 1998). Another is the sequence GAGAG, a known binding site for two Drosophila proteins, GAGA factor and Pipsqueak (Soeller *et al.* 1993; Lehmann *et al.* 1998). The sequence GAGAG has been shown to be important for the pairing-sensitive silencing activity of the *iab-7* PRE (Mishra *et al.* 2001). In that case, the authors were able to show that mutations in *Trl*, the gene that encodes GAGA factor, reduce the silencing activity of the *iab-7* PRE (Hagstrom *et al.* 1997; Mishra *et al.* 2001). In contrast, *Trl* mutations do not affect the eye color of flies with the 139-bp *engrailed* PSE (data not shown). The GAGAG sequence has also been found to be important for the silencing activity of the *Mcp* and *bxd* PREs (Busturia *et al.* 2001; Hodgson *et al.* 2001). Biochemical and genetic evidence suggest a role for both GAGA factor and Pipsqueak in PcG silencing (Horard *et al.* 2000; Busturia *et al.* 2001; Hodgson *et al.* 2001).

It is unclear why pairing-sensitive silencing occurs at only ~60% of insertion sites, although it presumably involves the nearby flanking sequences, which might either contribute to or prevent the effect. Here we have shown that addition of binding sites for the enhancer protein Glass, while it darkened the eye color of heterozygous flies, did not change the percentage of lines with pairing-sensitive silencing (Figures 1 and 3). This suggests two things. First, it is unlikely that there is a class of lines with strong mini-*white* repression in heterozygotes that we failed to recover using the *CaSpeR* vector alone, and second, pairing-sensitive silencing is not reduced significantly by the presence of this activator. In fact, curiously, pairing-sensitive silencing was more efficient with the GBS vector, in that more lines had complete silencing (data not shown). These results differ somewhat from those obtained by Mishra *et al.* (2001), where a greater percentage of lines with pairing-sensitive silencing were obtained using a vector without an eye enhancer than with the enhancer. However, those authors used a different eye enhancer (from the *white* gene), so the results are not directly comparable.

Our data suggest that mutations in any one of five protein-binding sites reduce the level of pairing-sensitive silencing, although they do not reduce it to zero. Transgenic lines with *CaSpeR* alone rarely if ever show pairing-sensitive silencing. In contrast, in lines with the 139-bp PSE carrying mutations in the Pho, GAGAG, site 2, or NC1 sites, the percentage of lines with pairing-sensitive silencing was reduced from ~60 to 10%. Even the synthetic constructs containing only the Pho, GAGAG, and site 2 sequences gave a low number of lines with pairing-sensitive silencing. These data suggest that flanking genomic sequences can contribute to the activity of an impaired PSE. The interplay between PSEs and flanking genomic DNA has not been thoroughly explored. We previously showed that a PSE-containing transgenic line with the insertion at a chromosomal site that did not allow pairing-sensitive silencing could be converted into a pairing-sensitive line by duplication of the transgene (Kassis *et al.* 1991), effectively putting two PSEs near each other in the genome. These data suggested that a flanking genomic PSE may be an important component of pairing-sensitive silencing. Aside from one example (Whiteley *et al.* 1992), this idea has not been further investigated.

Here we have shown that *engrailed* PSEs can work as PREs in a *bxd-Ubx-lacZ* reporter. We believe that this activity is significant, but offer the caveat that this reporter may contain a weak PRE (Müller and Bienz 1991). Thus, the *engrailed* PSE may not be able to stably recruit or maintain Polycomb-group protein complexes by itself, but may nonetheless be an essential component of PRE function (for a complete discussion see Kassis 2002). Interestingly, in tissue culture cells, the 181-bp PSE appears to be bound only by Psc and not by Pc or Ph (Strutt and Paro 1997). This Psc binding might potentiate the action of a weak Pc- and Ph-binding PRE present in the *bxd-Ubx-lacZ* vector. A functional distinction between pairing sites and PREs has also been sug-

gested by Hodgson *et al.* (2001). Clearly, further studies using different PRE vectors, as well as functional studies of the role of these PSEs at the *engrailed* locus itself, are required to fully understand the biological role of *engrailed* PSEs.

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