Regulatory Regions of the Homeotic Gene *proboscipedia* Are Sensitive to Chromosomal Pairing

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ABSTRACT

We have identified regulatory regions of the homeotic gene *proboscipedia* that are capable of repressing a linked *white* minigene in a manner that is sensitive to chromosomal pairing. Normally, the eye color of transformants containing *white* in a *P*-element vector is affected by the number of copies of the transgene; homozygous flies have darker eyes than heterozygotes. However, we found that flies homozygous for select *pb* DNA-containing transgenes had lighter eyes than heterozygotes. Several *pb* DNA fragments are capable of causing this pairing sensitive (PS) negative regulation of *white*. Two fragments in the upstream DNA of *pb*, 0.58 and 0.98 kb, are PS; additionally, two PS sites are located in the second intron, including a 0.5-kb region and 49-bp sequence. This phenotype is not observed when two PS sites are located at different chromosomal insertion sites (in *trans*-heterozygous transgenic animals), indicating that the *pb*-DNA-mediated repression of *white* is dependent on the pairing or proximity of the PS regions. The observed phenomenon is similar to transvection in which certain alleles of a gene can complement each other, but only when homologous chromosomes are paired. Interestingly, the intronic PS regions contain positive regulatory sequences for *pb*, whereas the upstream PS sites contain *pb* negative regulatory elements.

IN *Drosophila melanogaster*, the specification of segmental identities in the body plan is controlled by the products of the homeotic genes (reviewed in DUNCAN 1987; KAUFMAN *et al.* 1990; MORATA 1993). Misregulated expression of these genes causes cells in certain segments to adopt the identity of cells normally found in other segments. Therefore, the accumulation patterns of the protein products of homeotic genes must be precisely controlled. Initial expression of homeotic genes is under the control of several groups of genes, collectively referred to as segmentation genes (reviewed in AKAM 1987; INGHAM 1988). Maintenance of the specific expression patterns of homeotic genes is achieved by autoregulation (BIENZ and TREMML 1988; KUZIORA and MCGINNIS 1988; CHOUINARD and KAUFMAN 1991) and by *trans*-acting factors, including members of the *Polycomb* group (Pc-G) and the *trithorax* group (trx-G) (PARO 1990; KENNISON 1993). The trx-G genes are required for activation, whereas the Pc-G genes are needed for repression of the homeotic genes outside of their normal expression domains. The gene products of members of these two gene groups appear to act in multimeric protein complexes and may function by affecting chromatin structure (PARO 1990; FRANKE *et al.* 1992; KENNISON 1993; MARTIN and ADLER 1993).

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Maintenance of gene expression patterns of some homeotic genes such as *Ultrabithorax* (*Ubx*) and *Abdominal-B* (*Abd-B*) is thought to be achieved by long range repression (MULLER and BIENZ 1991; BUSTURIA and BIENZ 1993; CHAN *et al.* 1994). It has been suggested that this mechanism may be a general principle applicable to a majority of homeotic genes (MULLER and BIENZ 1991; BUSTURIA and BIENZ 1993). According to this model, each homeotic gene has the potential to be activated in a broad region along the body axis, but *cis*-acting silencer elements mediate a restriction of this expression to specific domains. Silencer regions appear to be capable of functioning at significant distances from their associated promoters, for example, a *Ubx* silencer is located over 40 kb from the *Ubx* promoter (MULLER and BIENZ 1991). Proteins bound to these negative regulatory regions are expected to confer a heritable state of repression. Recent studies suggest that some Pc-G genes act through silencer elements in the *Ubx*, *Abd-B*, and *Sex combs reduced* (*Scr*) genes to maintain spatial boundaries of gene expression throughout development (MULLER and BIENZ 1991; BUSTURIA and BIENZ 1993; GINDHART and KAUFMAN 1995).

An interesting observation regarding the actions of the Pc-G and trx-G gene products is that some of them appear to interact (directly or indirectly) with DNA regions that are "pairing sensitive" (PS) (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995). PS regions (first described in KASSIS *et al.* 1991) are capable of mediating repression of a *white* minigene located in a

P-element vector in a manner that is sensitive to chromosome pairing. Normally flies that are homozygous for a *P* element containing a *white* minigene have darker eyes than heterozygotes (KLEMENZ *et al.* 1987; PIRROTTA 1988). However, when PS sites are contained in these *P*-element vectors, this dosage-dependent effect is not observed; homozygotes carrying these constructs have lighter eyes than heterozygotes. This PS phenomenon is similar to transvection effects in which certain alleles of a gene can complement each other but only when the homologous chromosomes are able to pair (reviewed in JUDD 1988; ASHBURNER 1989; TARTOF and HENIKOFF 1991; WU 1993). It is postulated that enhancers on one homolog affect the promoter sequences on the other homolog. These allelic interactions are observable because homologous chromosomes of Drosophila are paired in diploid nuclei (METZ 1916; KOPCZYNSKI and MUSKAVITCH 1992; HIRAOKA *et al.* 1993). Recently, discrete regions of DNA from several loci have been found to exhibit PS effects (KASSIS *et al.* 1991; HAZELRIGG and PETERSON 1992; CHUNG *et al.* 1993; FAUVARQUE and DURA 1993; KASSIS 1994; GINDHART and KAUFMAN 1995). The PS regions of *Scr* and *polyhomeotic* (*ph*) appear to interact with some of the Pc-G and trx-G gene products (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995). In addition, a regulatory fragment of *Ubx* has been found to both inactivate *white* in a *P*-element construct and contain elements that form a complex with the products of the Pc-G genes (CHAN *et al.* 1994). These observations suggest that large multimeric protein complexes consisting of the Pc-G and trx-G proteins may interact with PS sites to maintain the expression patterns of these genes by altering chromatin structure (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995). However, some PS regions, such as those found in the *engrailed* (*en*) locus, do not appear to be influenced by genes of the Pc and the trx groups. For this reason, a more general model for the function of PS sites has been proposed for *en* (KASSIS *et al.* 1991; KASSIS 1994). The PS elements at the *en* locus are thought to be bound by protein complexes that function to mediate interactions between distant enhancer sequences and the *en* promoter. Collectively, these observations suggest that understanding the nature of PS sequences has the potential to yield valuable information about silencer elements, promoter-enhancer interactions, and consequently about homeotic gene regulation.

Recently, we discovered that sequences contained in a *proboscipedia* (*pb*) minigene are capable of suppressing a *white* marker gene located in a *P*-element vector. In this study, we set out to identify the PS regions of *pb* and to test if they are important to the function of the *pb* gene. We report the identification of discrete regions of the *pb* locus that are able to repress the *white* gene in a *P*-element vector in a manner that is sensitive to homolog pairing. These regions do not appear to be regulated by several members of the Pc-G and trx-G. Some PS sites contain positive regulatory elements, whereas others appear to function as negative regulators of *pb*, suggesting that these regions are important for the developmental regulation of *pb*. We discuss the implications of these findings in terms of a general model for how PS sites function.

MATERIALS AND METHODS

Generation of fusion gene constructs: The *P*-element vectors used in this analysis include HZR, pbZR, and pbZRH. HZR contains an hsp70 promoter-*lacZ* fusion gene and a *white* minigene as a marker for transformation (GINDHART *et al.* 1995). The hsp70 promoter in HZR was replaced with a 0.60-kb basal *pb* promoter to create pbZR (KAPOUN and KAUFMAN 1995). The pbZRH construct was generated by replacing the hsp70 promoter of HZR with a 1.3-kb *pb* fragment (−98 bp to + 1.2 kb from the *pb* transcription start site). To create this construct, one of the two *Pst*I restriction enzyme sites contained in HZR was eliminated by performing a *Pst*I partial digestion and blunt-ending with T4 DNA polymerase. The modified vector containing a unique *Pst*I site in the hsp70 leader sequences was digested with *Pst*I, and the cohesive ends were made blunt. This vector was digested with *Kpn*I (to remove most of the hsp70 promoter sequences) and ligated to the 1.3-kb *Kpn*I-*Hin*dIII (the *Hin*dIII end was made blunt with Klenow) *pb* fragment.

Various genomic fragments of *pb* DNA (Figure 1B) from λA10 (SCOTT *et al.* 1983) were inserted into one or more of the *P*-element vectors described above. The 9.6-kb region includes *pb* DNA from −7.3 kb of the transcription start site to a *Bam*HI site located in second exon. The 5.7-kb *Bam*HI-*Sal*I fragment includes a subregion of the 9.6-kb fragment that contains the DNA that is 5′ to the *Sal*I site in the *z2* locus. The 1.5-kb fragment extends from the *Sal*I site in *z2* to the *Kpn*I site located at −98 bp from the *pb* start site. This region was divided at the *Pst*I site (located at −0.7 kb) to generate the 0.98- and 0.58-kb fragments. The 0.60-kb region extends from the *Nco*I site located in the first exon to the *Hin*dIII site in the first intron. Evolutionarily conserved *pb* second intron sequences (RANDAZZO *et al.* 1991) are located in the 2.1-kb *Bam*HI-*Sal*I fragment. The 0.8- and 0.5-kb fragments are subregions of this sequence. The r1r2 sequence is a 49-bp synthetic oligonucleotide that was designed from a block of sequence identity located within the conserved region of *pb*'s second intron: 5′GGCCGCGAATTCTGTGCCGGCAAAGATCTGTTATTTGCGATCATTTGTAACAATTTTCGAGGC. r1r2 was created with an internal *Eco*RI site (underlined in above sequence) and *Not*I cohesive ends to facilitate subcloning. A 32-bp motif that is contained in r1r2 is repeated in another region of the second intron conserved sequences (described in KAPOUN and KAUFMAN 1995). Finally, all of *pb*'s first intron sequences are contained in the 1.9-kb *Nco*I-*Bam*HI fragment. Immunological staining patterns generated from the following fusion genes are reported in KAPOUN and KAUFMAN (1995): pbZR, 2.1+pbZR, 2.1+HZR, 1.9+2.1+pbZR, 0.8+pbZR, 0.5+pbZR, and r1r2+pbZR.

Minigene constructs: The ΔSalKpn and ΔPst minigenes used in this analysis (Figure 2) are modifications of the SPL *pb* minigenes described in KAPOUN and KAUFMAN (1995). Details concerning the construction of the minigenes are available upon request.

Fly stocks: Flies were cultured at 25° on standard Drosoph-

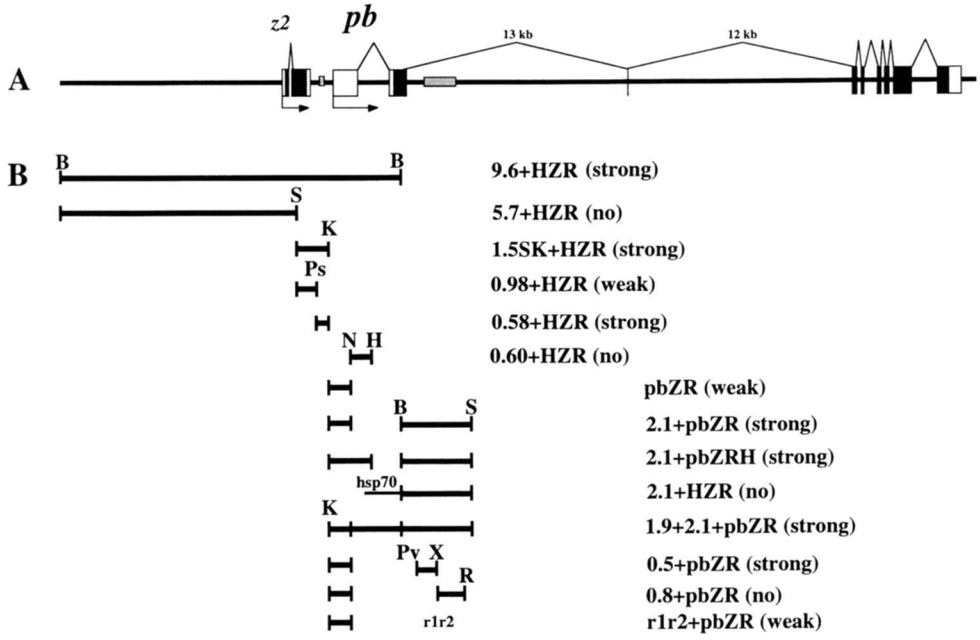

FIGURE 1.—Genomic fragments of *pb* used in this analysis. (A) The molecular organization of the *pb* locus is diagramed. Detailed structural analysis can be found in CRIBBS *et al.* (1992). *pb* is transcribed from left to right (arrow). Coding and noncoding exons are represented by black and white boxes (respectively), and the stippled boxes represent DNA that is conserved between *D. melanogaster* and *D. pseudoobscura*. A 15-bp alternatively spliced microexon is shown as a black vertical line. *pb* is closely flanked by an unrelated gene, *z2*, which can be deleted from the genome without any discernable affects (PULTZ *et al.* 1988). (B) *pb* DNA subclones from λA10 (SCOTT *et al.* 1983) were inserted into one or more of the following vectors: HZR, pbZR, and pbZRH (see MATERIALS AND METHODS). Constructs are named with + or − between the size of the cloned *pb* fragment and the *P*-element vector. + and − refer to the orientation of the cloned fragment with respect to the *lacZ* fusion gene. +, *pb* DNA is inserted in the same orientation as it resides in the endogenous *pb* locus; −, inverse orientation. The words "strong", "weak", and "no" refer to the degree of PS conferred by each *pb* DNA fragment. The following restriction enzyme sites are used: B, *Bam*HI; S, *Sal*I; K, *Kpn*I; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*I; X, *Xho*I; R, *Eco*RI. The basal hsp70 promoter is referred to as hsp70, and r1r2 indicates a synthetic oligonucleotide that was inserted upstream of the pbZR fusion gene.

ila media fortified with active dry yeast. Minigene transgenic stocks were established in *Ki* pb^5 p^p/TM3, *Sb* and *Ki* pb^5 p^p/TM6B, *Hu Tb* backgrounds (LINDSLEY and ZIMM 1992; FlyBase 1994). The gamma-induced pb^5 allele is a protein null (KAUFMAN 1978; CRIBBS *et al.* 1992). Homozygous pb^5 flies were scored both by the presence of the Sb^+ and the *Ki*/*Ki* phenotypes. pb^5/pb^5 third instar larvae were identified by the Tb^+ phenotype. Adult *pb* homeotic transformation rescue experiments were performed with one copy of the minigene unless otherwise noted.

***P*-element transformation:** Germ line transformation (ROBERTSON *et al.* 1988) was performed using a 0.5-mg/ml solution of each minigene. Transformants were selected by the rescue of the $white^-$ phenotype in the recipient flies. Multiple independent transformant lines were generated for each *P*-element construct as indicated in Table 1. Only transformant lines with insertions on the first and second chromosomes were tested for PS in this analysis. This eliminated confounding affects caused by the possibility of selective insertion of the minigenes into the *pb* locus (located on the third chromosome), which has been reported to occur at some loci (KASSIS *et al.* 1992; KASSIS 1994).

Immunological staining and scanning electron microscopy: Imaginal disc staining was performed as described in PATTATUCCI and KAUFMAN (1991). The anti-PB rabbit polyclonal antisera used for imaginal tissues were generated against the C-terminal region of PB (described in CRIBBS *et al.* 1992). FAB′ goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies were used for third instar larval stage staining (1:50 dilution, Protos Immuno Research). Analysis and photography of stained imaginal tissues were performed using a Zeiss Axiophot with Nomarski optics. All discs were photographed at 200× magnification unless otherwise indicated.

As with other minigene investigations (CASTELLI-GAIR *et al.* 1992; KAPOUN and KAUFMAN 1995), we observed variability among transformant lines and among individuals in each line. The expression patterns and phenotypes presented here represent what was observed in a majority of the animals.

Scanning electron microscopy was performed as described in MERRILL *et al.* (1987). *Ki* pb^5 p^p/*Ki* pb^5 p^p or *Ki* pb^5 p^p/TM3, *Sb* minigene transgenic adults were stored in 70% ethanol until they were prepared for microscopy.

Eye color assays: All adults examined for eye color and pigmentation patterns were 9 days of age. In each case, heterozygote and homozygote flies of the same sex were compared. The *pb* regions tested were classified into one of three groups, PS, weak PS, and not PS, based on the following criteria: PS, 60% of the transformant lines of a given construct exhibit PS and often some lines demonstrate complete repression of *white* as homozygotes; weak PS, 20–60% of the lines exhibit PS and usually no lines exhibit complete repression of *white* as homozygotes; and not PS, <20% of the lines show PS and no lines completely repress *white*. Other groups have reported similar frequencies for PS regions (*en*, 57–100%, and *ph*, 70%) and weak PS regions (*en*, 25%) (FAUVARQUE and DURA 1993; KASSIS 1994). The percentage in the not-PS group was chosen to account for genomic position effects due

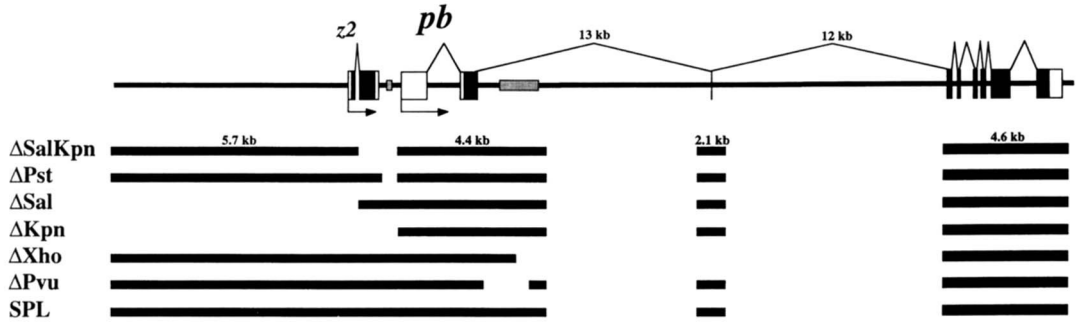

FIGURE 2.—Structure of *pb* minigenes. The thick lines drawn under the *pb* locus (described in Figure 1A) represent the *pb* regions that are contained in the transgenes. Minigenes ΔSalKpn and ΔPst were analyzed in this report; ΔSalKpn contains a −1.6-kb to −98-bp deletion that removes the 1.5-kb PS region, and ΔPst contains a −0.7-kb to −98-bp deletion that removes the 0.58-kb PS fragment. For reference, the structure of other *pb* minigenes (ΔSal, ΔKpn, ΔXho, ΔPvu, and SPL) (KAPOUN and KAUFMAN 1995), which are referred to in the text, are indicated.

to the site of *P*-element insertion, which have been reported to occur at low frequencies (15%) (HAZELRIGG *et al.* 1984; LEVIS *et al.* 1985a). Eyes were photographed using a Nikon 35 mm camera with Kodak Gold 100 ASA film.

Pc-G and trx-G alleles tested in combination with the *pb* PS DNA: The following alleles were tested for a genetic interaction with the *pb* PS sites: Pc-G alleles include $E(z)^{s1}$, $E(z)^{s6}$ (WU *et al.* 1989; JONES and GELBART 1990; PHILLIPS and SHEARN 1990), Pc^{3} (LEWIS 1978; DUNCAN and LEWIS 1982), Scm^{D1} (BREEN and DUNCAN 1986); and trx-G alleles include $ash\text{-}1^{RF605}$, $ash\text{-}1^{B1}$ (SHEARN *et al.* 1987; SHEARN 1989), brm^{2}, brm^{5}, dev^{1}, dev^{2}, kto^{1}, kto^{3}, $l(3)87Ca^{E2}$, $l(3)87Ca^{12}$, mor^{1}, mor^{2}, osa^{1}, osa^{2}, skd^{1}, skd^{2} (KENNISON and TAMKUN 1988), trx^{E2} (INGHAM and WHITTLE 1980), trx^{3}, urd^{2}, vtd^{3}, vtd^{5} (KENNISON and TAMKUN 1988). Additional genotypes tested were $brm^{2}trx^{E2}$, $brm^{2}Pc^{4}$, and $Pc^{4}trx^{E2}$ (supplied by J. KENNISON). Three independent PS *pb* minigene lines, ΔSal, ΔXho, ΔPvu (Figure 2) KAPOUN and KAUFMAN 1995), were tested with each of the alleles listed above. Minigenes ΔSal and ΔXho contained the 0.58-kb, 0.5-kb, and the r1r2 PS *pb* fragments, whereas ΔPvu included only the 0.58-kb PS region (Figures 1B and 2). The eye color of ΔPvu homozygous transformants is white; the eye colors of ΔSal and ΔXho homozygotes are different shades of pale orange. Transgenic w^{1}; P{w^{+}}/SM5; mutant/Balancer males were crossed to female w^{1}; P{w^{+}}/SM5 flies. The eye color of two sets of progeny classes was compared: w^{1}; P{w^{+}}/SM5; nonmutant and w^{1}; P{w^{+}}/SM5; mutant, and w^{1}; P{w^{+}}/P{w^{+}}; nonmutant and w^{1}; P{w^{+}}/P{w^{+}}; mutant flies.

RESULTS

We recently completed a functional analysis of a series of *pb* minigenes (KAPOUN and KAUFMAN 1995). These minigenes lack most of the large intronic region of *pb* (shown in Figure 2) except for some conserved regulatory DNA located in the second intron. In the course of these studies, we noticed that sequences contained in the *pb* minigenes were capable of repressing the *white* minigene located in a *P*-element vector (pCaSpeR). Normally, the eye color of transformants harboring two copies of the *white* minigene (in pCaSpeR) is more pigmented than those with one copy (Figure 3, A and B) (PIRROTTA 1988). This dosage-dependent effect was not observed in a majority of the *pb* minigene lines that could be examined in the homozygous condition. Usually, homozygotes had a lighter or similar eye color intensity relative to heterozygotes (Figure 3, compare D with C). In one minigene line, complete repression of *white* was seen in the homozygous condition (Figure 3, compare F with E). In addition, a mottled pigmentation pattern of the eye was observed in many of the transgenic heterozygote animals. This pattern is also indicative of repression of the *white* minigene and is not observed in transformants harboring the *P*-element vector pCaSpeR alone (PIRROTTA 1988). These results suggest that *pb* minigene sequences can repress *white* expression and that repression is enhanced when transformants are made homozygous.

Discrete upstream and intronic regions of *pb* can mediate PS repression: To identify the region(s) of the *pb* locus responsible for the pairing sensitive (PS) repression, we examined the eye color of *P*-element transformant lines containing various *pb* DNA fragments (Figure 1B). Several fragments, both 5′ and 3′ to the *pb* transcription start site, were identified that could mediate PS repression of the *white* minigene. The results are summarized in Table 1 and described below. In this analysis, a DNA region was designated PS if any of the following conditions existed stably in transgenic animals that contained the fragment: homozygotes showed domains of *white* repression that were not observed in heterozygotes, but other regions of the eye had increased levels of pigmentation (patterned eyes) (Figure 4, C and D); the eye color of homozygotes and heterozygotes was similar (Figure 4, E and F); and homozygotes had lighter eyes than heterozygotes or showed complete repression of *white* in the homozygous condition (Figure 4, G and H). Some transgenic animals also displayed a mottled pattern of eye pigmentation as homozygotes (Figure 4, I and J). Because sometimes this mottling was subtle and could be subject to different interpretations, this condition was omitted from the PS assessment.

A 9.6-kb fragment that contains 7.3 kb of upstream *pb* DNA and downstream sequences up to and including

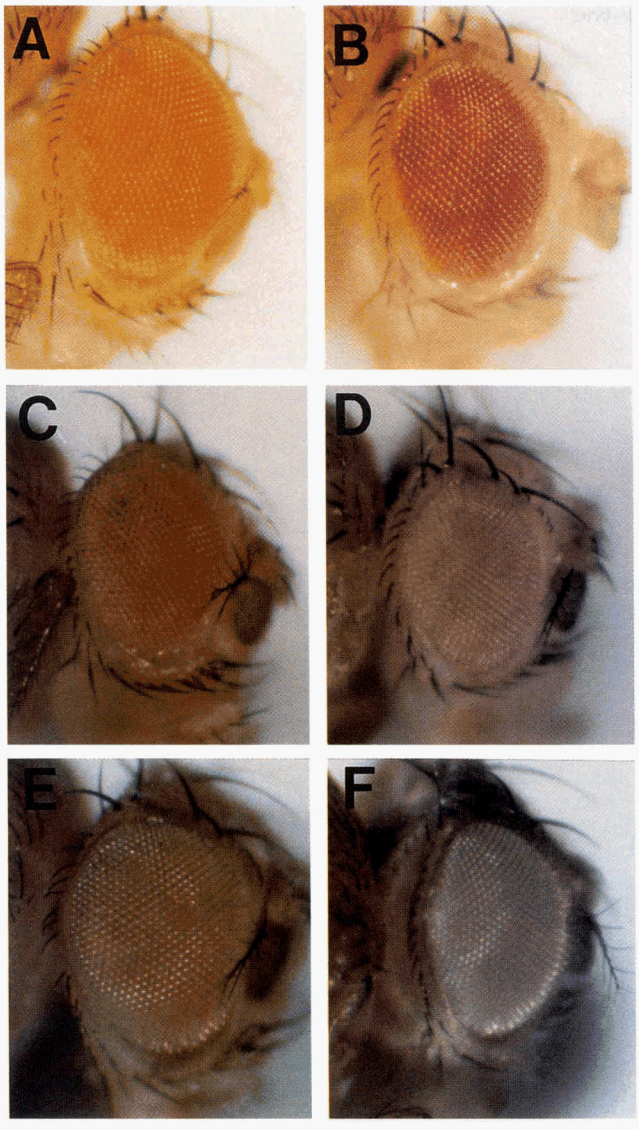

FIGURE 3.—*pb* minigenes mediate PS repression of *white*. (A and B) Representative transformant animals that exhibit the normal dosage sensitivity of the *white* minigene; (A) an animal with one copy of the *white* minigene (w^1; P{w^+}/ +) has lighter eyes than (B) an individual with two copies of the minigene (w^1; P{w^+}/P{w^+}). (C–F) Transformants harboring *pb* minigenes suppress *white* in a manner that is sensitive to the pairing of chromosomes. The eye color of a representative (D) homozygote (w^1; P{w^+, ΔXho}/P{w^+, ΔXho}) animal is similar to that of a (C) heterozygote (w^1; P{w^+, ΔXho}/ +). (F) A homozygous (w^1; P{w^+, ΔPvu}/P{w^+, ΔPvu}) animal shows complete repression of *white* compared with a (E) heterozygote (w^1; P{w^+, ΔPvu}/ +).

part of the second exon was first tested for the PS phenotype (Figure 1B). All transgenic 9.6+HZR and 9.6−HZR were PS (Table 1A and Figure 4, C and D). To further localize the sequences of *pb* that mediate PS, this region was dissected into three fragments, 5.7, 1.5, and 0.6 kb (Figure 1B). Transformants that contain the 5.7- and 0.6-kb sequences were not PS (Table 1A). However, the 1.5-kb region between the *z2* gene and −98 bp from the *pb* transcription start site did mediate the PS phenotype (Table 1A and Figure 4, E and F). This result was seen when the region was placed in either orientation with respect to the *white* gene in the *P*-element vector. Next, two subclones of this region were tested for PS (Figure 1B), which resulted in the identification of a 0.58-kb strong PS region and a 0.98-kb weak PS fragment (Table 1A and Figure 4, G–J). Interestingly, the 1.5SK−HZR vector that places the 0.58-kb PS site closer to the *white* gene conferred a stronger PS phenotype than the 1.5SK+HZR construct (64% of the 1.5SK−HZR and 36% of the 1.5SK+HZR transformant lines were PS). These results show that a discrete 0.58-kb fragment located in the 5′ region of *pb* is capable of mediating PS repression of the *white* minigene and that the degree of PS mediated by this fragment may be dependent on its distance from *white*.

To test whether other PS regions exist in the *pb* locus, we examined the eye color of transgenic lines harboring *P* elements containing second intron sequences linked to *pb* promoter-*lacZ* fusion genes (Figure 1B). A 2.1-kb second intron fragment was demonstrated to be PS when placed upstream of the *pb* promoter. Among the lines that contained this fragment linked to a *pb* promoter, 60% (15/25 lines) exhibited a PS phenotype (Table 1B). Interestingly, when the 2.1-kb fragment was combined with the hsp70-*lacZ* transgene, a PS phenotype was not observed (Table 1B). This implies that an interaction between the *pb* promoter and the intronic DNA may be important for the *pb* DNA-mediated negative regulation of *white*. Transgenic flies bearing the *pb* promoter-*lacZ* fusion transgene without the second intron sequences (pbZR) displayed a very weak PS effect (Table 1B), suggesting that the *pb* promoter may contain some elements that can generate PS repression. Subregions of the 2.1-kb intron two region were also tested (Figure 1B). A 0.8-kb subfragment did not mediate PS (Table 1B), whereas a 0.5-kb sequence was able to confer the PS phenotype in 2/2 lines (most other lines were homozygous lethal and could not be tested). In addition, a 49-bp synthetic oligonucleotide designed from conserved *pb* regulatory DNA demonstrated weak PS in homozygotes. Finally, the 2.1−pbZRH transgenic lines, which contain the 0.5-kb fragment closer to *white*, showed a stronger PS affect than the 2.1+pbZRH lines (64% *vs.* 40%). These results support the observation that the 0.5-kb region is PS and suggest that its effect on *white* are in part dependent on proximity.

In some transgenic lines containing the *pb* DNA fragments diagramed in Figure 1B, we noticed that heterozygous animals displayed a mottled or patterned eye color phenotype. For example, 67% (6/9) of heterozygous 9.6+HZR and 9.6−HZR adults showed mottling (Table 1A). In addition, 82% of 2.1−pbZRH and 67% of 1.9+2.1+pbZR transgenic lines demonstrated this type of *white* repression as heterozygotes (Table 1B). In contrast, other transgene lines that contain PS frag-

TABLE 1

Pairing sensitive summary

Constructs[a]	PM total[b]	PM het[c]	PS[d]	W[e]	PS[f]
		A. Upstream PS regions			
9.6+HZR	5/7 (71)	5/7 (71)	3/3 (100)	Y	Y
9.6−HZR	1/2 (50)	1/2 (50)	1/1 (100)	N	Y
5.7+HZR	3/13 (23)	3/13 (23)	1/6 (17)	N	N
1.5SK+HZR	6/15 (40)	0/15 (0)	4/11 (36)	Y	W
1.5SK−HZR	7/17 (41)	4/17 (24)	7/11 (64)	Y	Y
0.58+HZR	3/12 (25)	1/12 (8)	9/9 (100)	Y	Y
0.98+HZR	5/15 (33)	3/15 (20)	2/7 (29)	N	W
0.60+HZR	1/16 (6)	1/16 (6)	1/9 (11)	N	N
		B. Intron 2 and promoter PS regions			
pbZR	11/28 (39)	9/27 (33)	2/10 (20)	N	W
2.1+pbZR	6/16 (38)	3/16 (19)	5/8 (63)	N	Y
2.1+pbZRH	4/16 (25)	3/16 (19)	2/5 (40)	Y	W
2.1−pbZRH	14/17 (82)	14/17 (82)	7/11 (64)	Y	Y
1.9+2.1+bpZR	6/9 (67)	6/9 (67)	1/1 (100)	N	Y
2.1+HZR	4/14 (29)	4/14 (29)	0/3 (0)	N	N
0.8+pbZr	1/6 (17)	1/6 (17)	0/4 (0)	N	N
0.5+pbZR	3/9 (33)	2/9 (22)	2/2 (100)	Y	Y
rlr2+pbZR	4/12 (33)	3/12 (25)	3/7 (43)	N	W

[a] *P*-element constructs containing *pb* DNA insertions. PS suppression of *white* is not observed in transformants carrying the HZR vector without insert DNA (GINDHART and KAUFMAN 1995).

[b] Number of total lines that show patterned (P) or mottled (M) eyes as heterozygotes and or homozygotes. Percents are included in parentheses.

[c] Number of lines that showed patterned or mottled eyes as heterozygotes (het). Percents are included in parentheses.

[d] Number of lines that were demonstrated to be PS. The number of total lines examined is less than the totals in columns one and two because some transformant lines were homozygous lethal due to the influence of flanking DNA or the *P* element inserted into a balancer chromosome that contained a homozygous lethal mutation. Percents are included in parentheses.

[e] W, white eye color in homozygous transformant animals; Y, homozygotes (of any of the multiple independent lines) exhibited complete *white* suppression; N, homozygous lines never showed complete repression of *white*.

[f] Y, the *pb* DNA-containing construct was demonstrated to mediate the PS phentoype (see MATERIALS AND METHODS); W, weak PS effect observed; N, no PS effect observed.

ments have lower percentages of mottling and/or patterning as heterozygotes (0% of 1.5SK+HZR, 24% of 1.5SK+HZR, 22% of 0.5+pbZR and 19% of 2.1+pbZR). The possible significance of the observed *white* repression in heterozygotes is discussed below (see DISCUSSION).

Repression of *white* is dependent on pairing or proximity of the *P*-element insertions containing *pb* DNA: To determine whether *pb* DNA-mediated *white* repression is due to a dosage effect or to the proximity of the inserts, heteroallelic combinations were made using different insertion lines harboring the same PS region-containing transgene. All insertion lines tested were on the second chromosome. They were presumed to be in different chromosomal locations based on different pigmentation levels in the eye and varying PS phenotypes. Two different 1.5SK−HZR transformant lines (1.5SK−HZR#10 and 1.5SK−HZR#14) are shown in Figure 5. In both lines, homozygote transformants have lighter eyes than heterozygous (Figure 5, compare D and F with C and E). In contrast, P{1.5SK−HZR#10}/P{1.5SK−HZR#14} *trans*-heterozygote flies had darker eyes than those in either P{1.5SK−HZR#10}/ + or P{1.5SK−HZR#14}/ + heterozygote flies (Figure 5, compare G with C and E). These results suggest that the PS effects mediated by *pb* DNA are not a result of a dosage effect (having two copies of the P{w^+} transgene in the genome) and that the proximity of *pb* PS sites is necessary to achieve *white* repression.

***pb* PS regions do not interact genetically with Pc-G or trx-G alleles:** Because PS regions in other loci exhibit genetic interactions with some members of the Pc-G and/or trx-G genes (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995), we tested the possibility that lowering the dosage of individual members of these gene groups would modify the regulatory activities of

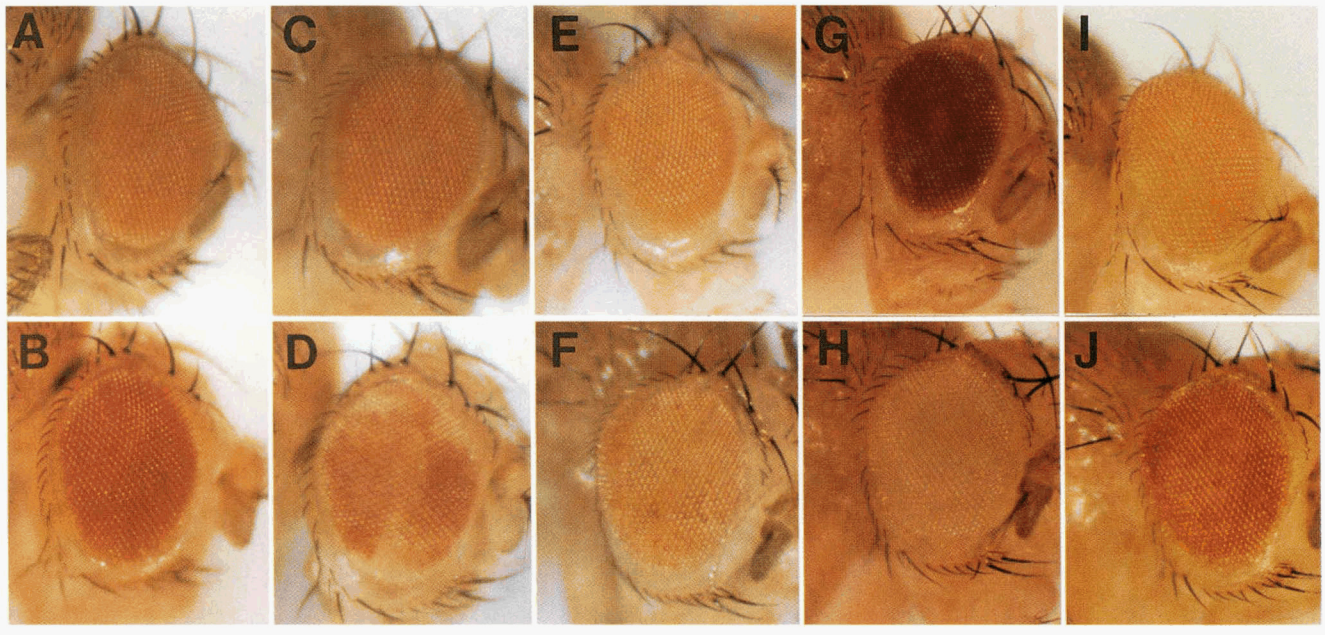

FIGURE 4.—Discrete *pb* DNA fragments show PS repression of the *white* minigene. (A and B) Representative transformant animals that exhibit the normal dosage sensitivity of the *white* minigene; (A) an animal with one copy of the *white* minigene (w^1; P{w^+}/ +) has lighter eyes than (B) an individual with two copies of the minigene (w^1; P{w^+}/P{w^+}). (C–J) Transformants bearing *pb* DNA fragments mediate *white* repression in a manner that is sensitive to chromosome pairing. (D) The patterned eye of a w^1; P{w^+, 9.6+HZR}/P{w^+, 9.6+HZR} animal compared with (C) a w^1; P{w^+, 9.6+HZR}/ + fly. (F) a w^1; P{w^+, 1.5+HZR}/ P{w^+, 1.5+HZR} animal has similar eye color (and eye color pigmentation is mottled) compared with a (E) w^1; P{w^+, 9.6+HZR}/ + animal. (H) The white eye of a w^1; P{w^+, 0.58+HZR}/P{w^+, 0.58+HZR} fly compared with a (G) w^1; P{w^+, 0.58+HZR}/ + animal. (J) The mottled eye of a w^1; P{w^+, 0.98+HZR}/P{w^+, 0.98+HZR} individual compared with a (I) w^1; P{w^+, 0.58+HZR}/ + animal.

the *pb* PS regions. Members of the trx-G are necessary for maintaining states of homeotic gene activation, whereas genes of the Pc-G are required to maintain repression of homeotic genes (PARO 1990; KENNISON 1993). If these gene products interact with the *pb* PS sites, an enhancement or repression of *white* expression could be expected in transformants carrying a *pb* PS region (in the transgene) in Pc-G or trx-G mutant backgrounds. For example, lowering the dosage of a Pc-G gene (negative regulator) may lead to an increase of *white* expression; conversely, lowering the dosage of a positively acting trx-G gene could lead to *white* repression. Transformants harboring *pb* PS DNA constructs were crossed to several different Pc-G and trx-G mutants (see MATERIALS AND METHODS). No significant changes in eye color were observed in heterozygote or homozygote transformant animals in mutant *vs.* nonmutant backgrounds. These results suggest that these Pc-G and trx-G gene products do not interact with the PS regions in the *pb* locus.

Minigenes lacking a PS region express *pb* ectopically in imaginal tissues: What is the significance of the PS regions to the regulation of *pb*? The second intron *pb* DNA fragments that are PS (2.1 kb, 0.5 kb, and r1r2) (Figure 1B and Table 1B) have been shown to contain positive *pb* enhancer elements (KAPOUN and KAUFMAN 1995). In contrast, a functional role for the upstream 1.5- and 0.58-kb PS fragments (Figure 1B and Table 1A) (both regions are upstream of −98 bp of the *pb* start site) has not been demonstrated. Recently, we have shown that a *pb* minigene lacking sequences upstream of −98 bp is ectopically expressed, but those containing 7.3 kb of upstream DNA are not (KAPOUN and KAUFMAN 1995). Because the upstream PS regions are deleted from the former minigene (but present in the latter), we wondered whether they are involved in the regulation of *pb*. To test this hypothesis, we deleted both the 1.5- and 0.58-kb PS fragments from independent *pb* minigenes (ΔSalKpn and ΔPst, respectively) (Figure 2). The SPL *pb* minigene (Figure 2) used to make the deletions completely rescues the *pb* homeotic phenotype and generates a wild-type pattern of PB accumulation, with the exception of central nervous system (CNS) expression (KAPOUN and KAUFMAN 1995).

Both deletion minigenes ΔSalKpn (1.5 kb deleted) and ΔPst (0.58 kb deleted) were crossed into a pb^5 null background to determine the extent of rescue and the pattern of transgenic *pb* expression. In *pb* null animals, the labial palps are transformed into first thoracic legs and the maxillary palps are reduced in size (Figure 6B). The mouthparts of P{w^+, ΔSalKpn}/ + ; pb^5/pb^5 or P{w^+, ΔPst}/ + ; pb^5/pb^5 adults were rescued (Figure 6, compare C and D with A), although sometimes small patches of aristal tissues were present on the lateral

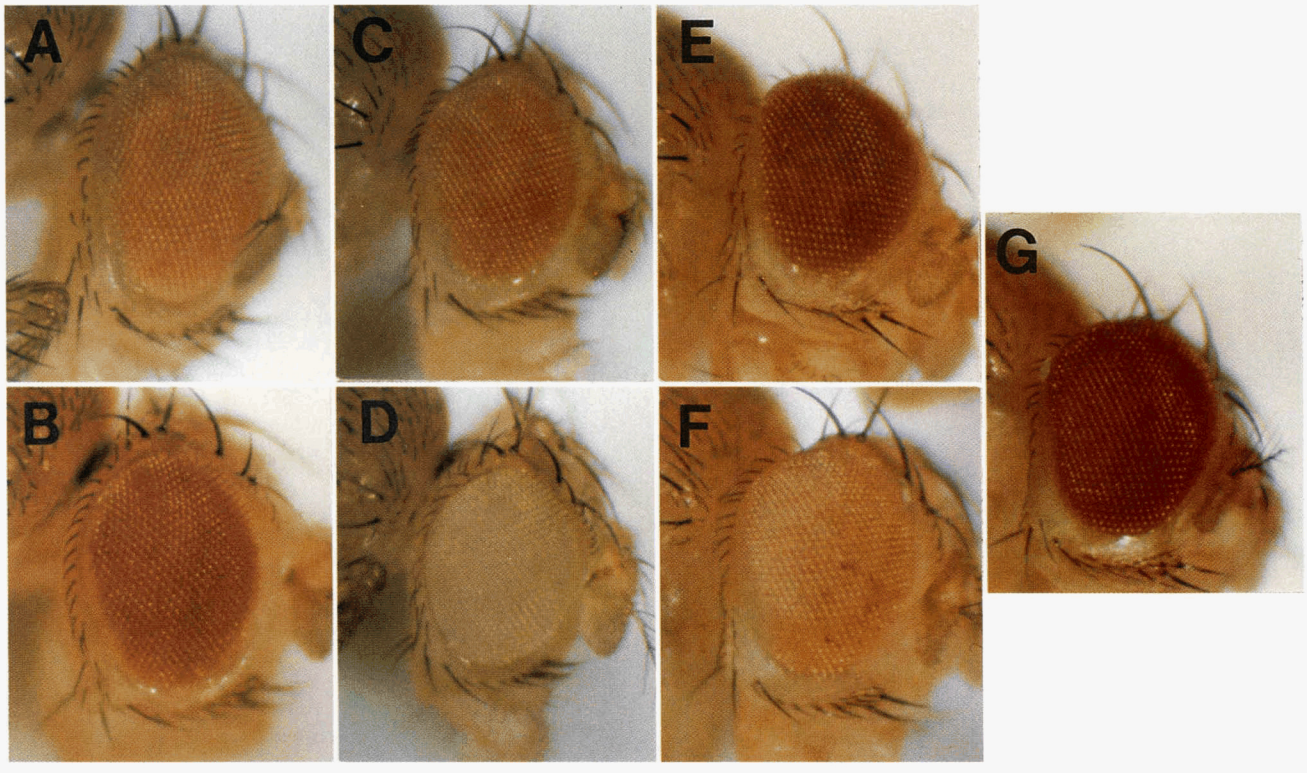

FIGURE 5.—Repression of the *white* minigene is dependent on the pairing of the *pb* PS fragments. (A and B) Representative transformant animals that exhibit the normal dosage sensitivity of the *white* minigene; (A) an animal with one copy of the *white* minigene (w^1; P{w^+}/ +) has lighter eyes than (B) an individual with two copies of the minigene (w^1; P{w^+}/P{w^+}). (C–F) Transformants bearing P{1.5SK–HZR} transgenes mediate *white* repression in a manner that is sensitive to the pairing of chromosomes. (D) w^1; P{1.5SK–HZR#10}/P{1.5SK–HZR#10} animals have white eyes compared with (C) w^1;P{1.5SK–HZR#10}/ + flies. (F) w^1P{1.5SK–HZR#14}/P{1.5SK–HZR#14} animals have lighter and mottled eyes compared with (E) w^1; P{1.5SK–HZR#14}/ + flies. (G) w^1P{1.5SK–HZR#10}/P{1.5SK–HZR#14} *trans*-heterozygote animals have darker eyes than those in either w^1P{1.5SK–HZR#10}/ + or w^1P{1.5SK–HZR#14}/ + individuals.

aspects of the labial palps (Figure 6C). In many cases, two copies of the minigenes reduced or eliminated the aristal phenotype.

The minigenes displayed a wild-type pattern of PB accumulation in embryos and in labial discs of third instar larvae. The *pb* expression pattern generated from ΔSalKpn and ΔPst during embryogenesis was similar to that of the SPL minigene, which contains an intact 7.3 kb of upstream *pb* DNA. However, the overall levels of PB in ΔSalKpn and ΔPst appeared slightly lower than that of SPL, especially in late embryos (data not shown). The labial discs in pb^5/pb^5 animals show no PB accumulation, and morphologically they resemble leg discs (Figure 7B), consistent with the labial palp-to-first thoracic leg transformation observed in *pb* null adults. In contrast, third instar larvae of P{w^+, ΔSalKpn}/ + ; pb^5/pb^5 or P{w^+, ΔPst}/ + ; pb^5/pb^5 animals accumulated significant levels of PB in the labial discs (Figure 7). Frequently, *pb* expression levels in these tissues were similar to that of wild-type animals (Figure 7, compare C with A), but sometimes they appeared slightly lower (Figure 7, compare D with A). CNS accumulation generated from ΔSalKpn and ΔPst was identical to the accumulation produced from a minigene that contains no deletions (SPL minigene) (KAPOUN and KAUFMAN 1995); *pb* expression was extremely weak in the embryonic ventral nerve cord and absent in most cells of the larval CNS (data not shown).

To determine whether transgenic *pb* expression is properly regulated outside of *pb*'s normal expression domain, we examined PB accumulation in other imaginal tissues that normally do not express *pb*. Both minigene lines accumulated PB in third instar leg discs (Figure 8, B and C), a domain where *pb* is not normally expressed (Figure 8A). In most lines, PB accumulation is localized to the central region of the leg discs (Figure 8, D and E). All transformant minigene lines showed this ectopic *pb* expression pattern (ΔSalKpn, 4/4, and ΔPst, 4/4), although in two lines of each, the accumulation was reduced (similar to Figure 8D) and sometimes apparent only in a few cells (not shown). No corresponding adult leg defects were detected in either heterozygote or homozygote minigene transgenic animals. In contrast, adult antennal transformations were observed in 63% (10/16 and 5/8) of the transformant lines for both the ΔSalKpn and the ΔPst minigenes (Figure 6, compare E with F). Transgenic minigene lines displayed a thickening of the arista, and sometimes

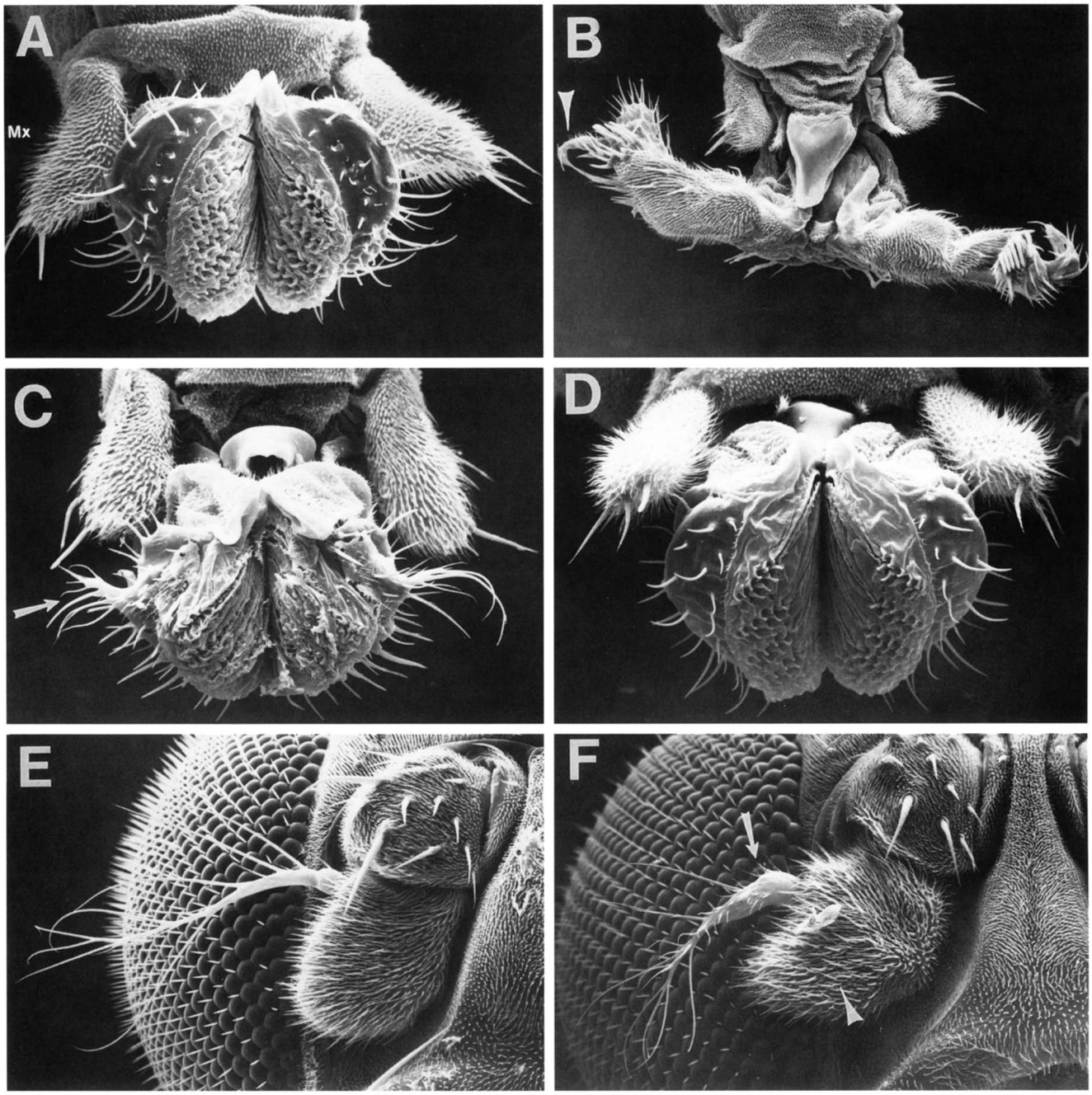

FIGURE 6.—Scanning electron micrographs of adult mouthparts and antennal structures. (A) Wild-type maxillary (Mx) and labial palps. The arrow points to a pseudotracheal row on the labial palps. (B) A pb^5/pb^5 (*pb* null) animal. Labial palps are transformed to first thoracic legs (arrowhead points to a claw), and maxillary palps are reduced in size. (C and D) P{w^+, ΔSalKpn}; pb^5/pb^5 and P{w^+, ΔPst}; pb^5/pb^5 rescued animals. The arrow (in C) points to aristal tissue that is sometimes present on the lateral aspects of the labial palps in these animals. (E) Wild-type antennal structures compared with those in a (F) transgenic P{w^+, ΔPst} animal. The arrow in F points to thickening of the arista, and the arrowhead is on the third antennal segment that is morphologically aberrant.

aberrant morphology is evident in the third antennal segment (Figure 6F). These antennal defects are indicative of ectopic PB accumulation in antennal discs of third instar larvae (KAPOUN and KAUFMAN 1995). Because minigenes with an intact 7.3 kb of *pb* upstream DNA do not express *pb* ectopically in these imaginal tissues (KAPOUN and KAUFMAN 1995), the 0.58-kb upstream PS region of *pb* appears to be required for proper negative regulation of the gene.

DISCUSSION

Identification of PS regions in the *pb* locus

We have identified discrete regions of the homeotic gene *pb* that can mediate PS repression of the *white*

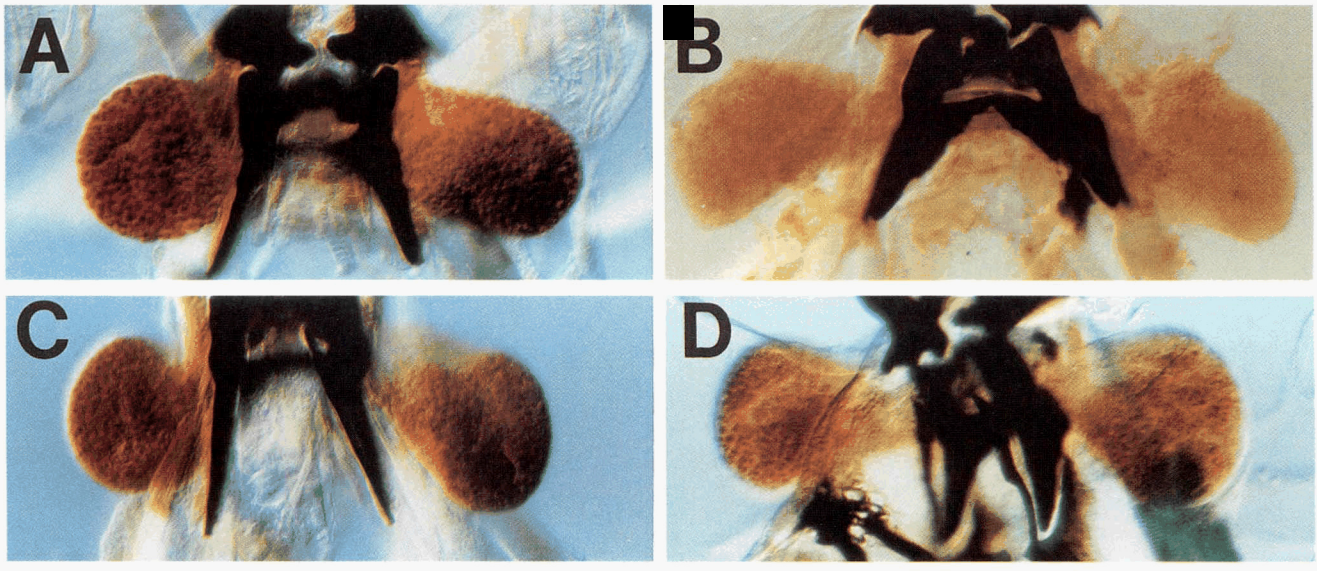

FIGURE 7.—PB accumulation in labial discs of third instar larvae. Labial discs from (A) wild-type and (B) pb^5/pb^5 animals stained with anti-*pb* antisera are depicted. *pb* null animals do not accumulate PB in these discs B. In contrast, the labial discs of (C) P{w^+, ΔSalKpn}/ + ; pb^5/pb^5 and (D) P{w^+, ΔPst}/ + ; pb^5/pb^5 animals show *pb* accumulation. The levels of PB in the labial discs in these transgenic animals are similar to wild type, although in some animals lower protein levels are observed D.

minigene in a *P*-element vector. Normally the eye color of transformants harboring the *white* minigene is dosage dependent, for example, homozygous flies have darker eyes than heterozygotes (KLEMENZ *et al.* 1987; PIRROTTA 1988). In contrast, animals homozygous for certain *pb* DNA-containing transgenes had lighter eyes than heterozygotes. Several *pb* DNA fragments were shown to mediate this repression of *white* (Figure 1B). A 0.58-kb strong PS sequence and a 0.98-kb weak PS region are located in *pb*'s upstream DNA. In addition, a 2.1-kb second intron region of *pb* was found to mediate a strong PS effect when linked to the basal *pb* promoter. Two subregions of the 2.1-kb fragment, a 0.5-kb (strong PS) and a 49-bp sequence termed r1r2 (weak PS), also showed PS repression of *white*. Because weak PS regions are adjacent to strong ones, it is likely that they are extensions of the strong sites, containing some elements required for PS but not enough to produce the full effect. When two PS regions are located at different chromosomal insertion sites (in *trans*-heterozygous PS region-containing transformant animals), the PS phenotype is not observed. Therefore, *pb* DNA-mediated negative regulation of *white* is dependent on the pairing or proximity of the PS regions.

Significance of PS regions to pb regulation

Both the 5′ and the intronic PS regions are important for *pb* regulation. When the upstream 0.58-kb PS region is deleted from a minigene that confers a *pb* expression pattern, ectopic PB accumulation is observed in third instar imaginal tissues, implying that this PS region is involved in the negative regulation of *pb*. Interestingly, this fragment contains a 60-bp stretch that is 92% identical to the corresponding upstream region from *Drosophila pseudoobscura* (RANDAZZO *et al.* 1991), suggesting that this region is important for controlling *pb* expression. Recently, we have shown that a *pb* minigene (ΔKpn, Figure 2) lacking all sequences upstream from −98 bp of the transcription start site expresses *pb* ectopically in imaginal tissues, whereas minigenes (SPL, ΔXho, and ΔPvu) with 7.3 kb of intact upstream DNA do not express this pattern (KAPOUN and KAUFMAN 1995). The ectopic expression in ΔKpn transformants appears to accumulate at higher levels than that observed in the ΔSalKpn and ΔPst lines (0.58-and 1.5-kb 5′ deletions, respectively), suggesting that additional more remote upstream regions are required for efficient repression of *pb* outside of its endogenous domain. These results imply that multiple regions upstream of *pb*, including the identified PS sites, act to silence ectopic *pb* expression.

The second intron 2.1-kb PS fragment of *pb* functions as an essential positive controlling region for the gene (KAPOUN and KAUFMAN 1995). Sequences included in this region are highly conserved between *D. melanogaster* and *D. pseudoobscura* (RANDAZZO *et al.* 1991). Moreover, the conserved intronic region contains enhancer elements that are essential for specifying the identity of the adult mouthparts. Two PS subregions of the 2.1-kb fragment, 0.5 kb and r1r2, contain *pb* enhancer elements. The 0.5-kb region confers a *pb*-like pattern in embryos and labial discs, and r1r2 confers labial disc expression (KAPOUN and KAUFMAN 1995). A 32-bp DNA motif that is conserved and repeated two times in the 2.1-kb *pb* fragment (RANDAZZO *et al.* 1991) is contained within r1r2, suggesting that it may be a labial disc en-

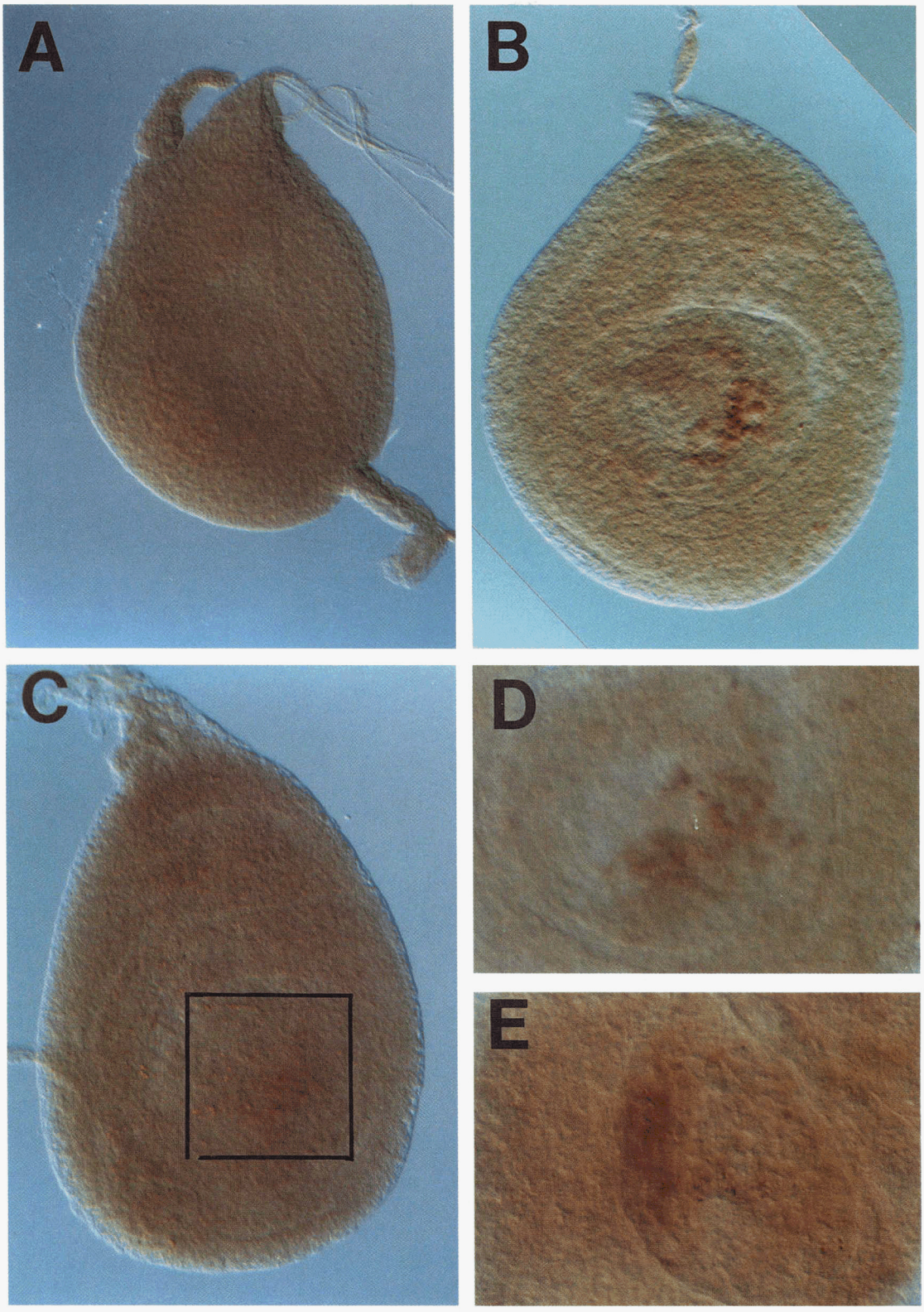

FIGURE 8.—*pb* is ectopically expressed in leg discs of transgenic third instar larvae. (A) PB is not detected in leg discs of wild-type animals stained with anti-PB antisera. In contrast, *pb* is expressed in the central most region (boxed-in area in C) of the leg discs in (B) P{w^+, ΔSalKpn}/ + and (C) P{w^+, ΔPst}/ + animals. (D and E) The central portion of leg discs for representative P{w^+, ΔPst}/ + (D) and P{w^+, ΔSalKpn}/ + (E) individuals at 400× magnification. Ectopic *pb* expression was observed in these transgenic animals in pb^5/ + or pb^5/pb^5 backgrounds.

hancer and/or a PS element. Interestingly, *pb*'s second intron regulatory region appears to confer proper expression when linked to the *pb* promoter but not with a heterologous (hsp70) promoter (KAPOUN and KAUFMAN 1995). This interaction parallels the PS phenotype observed for this region. When the 2.1-kb fragment was linked to an hsp70 promoter, a PS phenotype was not observed in transformants carrying this chimeric transgene. These results suggest that the interaction of the PS elements with the *pb* promoter is important for the *pb* DNA-mediated negative regulation of *white* and for the positive regulation of *pb* expression.

We propose the following model for *pb* regulation based on our results and the regulation of other homeotic genes (*Ubx* and *Abd-B*) (MULLER and BIENZ 1991; BUSTURIA and BIENZ 1993). The second intron regulatory region in combination with the *pb* promoter directs expression of *pb* in a broad domain in imaginal tissues, and restriction into *pb*'s expression pattern is carried out through the actions of multiple upstream silencer regions. The 5′ silencers are likely to be located both upstream and downstream of *z2* because independent minigenes that delete each of these regions generate ectopic leg and antennal PB accumulation (this report; KAPOUN and KAUFMAN 1995).

How do the putative upstream *pb* silencer regions function? Recent evidence in yeast suggests that chromatin conformation is involved in the function of silencers (APARICIO *et al.* 1991; PARK and SZOSTAK 1990). In addition, HOFMANN *et al.* (1989) reported that the yeast mating type silencers bind to the nuclear scaffold. Chromatin structure has also been implicated in the maintenance of homeotic gene expression in Drosophila. The gene products of the Pc-G and the trx-G loci are *trans*-regulators of homeotic genes, and they are thought to act in multimeric protein complexes that function to maintain homeotic gene expression in proper domains along the body axis (reviewed in PARO 1990; KENNISON 1993). The link to chromatin structure comes from the observation that a 37 amino acid domain of the *Polycomb* (*Pc*) gene product shares 65% similarity with HP1, a nonhistone heterochromatin-associated protein (PARO and HOGNESS 1991). Interestingly, silencer regions of *Ubx* and *Abd-B* appear to interact (either directly or indirectly) with the *Pc* protein (MULLER and BIENZ 1991; BUSTURIA and BIENZ 1993). In addition, negative regulatory sequences at the *Scr* gene have been shown to interact with the Pc-G and trx-G gene products (GINDHART and KAUFMAN 1995).

Because Pc-G and trx-G proteins also appear to interact with the PS sites of several loci (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995), we tested several members of these gene groups for an interaction with the *pb* PS sites. The PS regions of *pb* did not interact with any of the Pc-G or trx-G gene products tested, suggesting that the negative regulatory sequences contained in the *pb* PS sites are not mediated by members of these two gene groups. It is possible that other gene groups and/or additional members of the Pc-G or trx-G genes (those not tested here) are involved in maintaining *pb*'s expression domain. Additionally, multiple combinations of these *trans*-acting products may be required. Because a few homeodomain-containing gene products are only slightly altered in some Pc-G or trx-G mutant backgrounds and some, such as *labial, even-skipped,* and *empty spiracles,* are not altered (WEDEEN *et al.* 1986; BREEN and HARTE 1993; S. CHOUINARD, personal communication), other undiscovered or uncharacterized gene products may be involved in the maintenance of homeotic gene expression. Interestingly, the homeotic genes primarily expressed in the head (*labial, Dfd,* and *pb*) appear not to be significantly affected by the Pc-G or trx-G genes, although expression of *Dfd* is slightly altered in the CNS of *Pc* mutant embryos (WEDEEN *et al.* 1986). These observations may be explained by the fact that many of the Pc-G and trx-G genes were identified in screens that revealed dosage-dependent interactions with the homeotic genes expressed in the thorax (KENNISON and RUSSELL 1987; KENNISON and TAMKUN 1988). The upstream PS region of *pb* provides a reagent to screen for additional *trans*-regulators that are involved in maintenance of homeotic gene expression.

What are PS regions?

Model: We propose the following general model for how PS sites function. PS elements interact with multimeric protein complexes or aggregates involved in some aspect of gene regulation (positive or negative). Repression of *white* is a result of the protein complex (bound to the PS elements) interfering with the activity of the *white* promoter, and this interference can be independent of flanking genomic regions. When chromosomes are allowed to tightly pair, it is possible that binding of the protein components is enhanced resulting in more efficient repression in homozygous animals.

This model is based on several results that were observed in this study of the *pb* DNA-mediated negative regulation of *white,* and it shares some of the features that are common to other models that have been proposed for the functions of PS sites. The PS sites of *pb* appear to have diverse roles in the regulation of gene expression. For example, these regions appear to mediate both activation and repression of *pb.* In addition, it is possible that they act to bring regulatory sequences closer to the *pb* promoter. The PS elements that contain positive *pb* regulatory sequences (in the second intron) are positioned up to 4.5-kb away from the transcriptional start site. Moveover, the observation that these *pb* PS sites function in combination with the *pb* promoter and apparently not with a heterologous pro-

moter (with respect to *pb* function and PS repression) suggests that interactions between the *pb* PS site and the *pb* promoter region are important. The idea that PS sites can be involved in many aspects of gene regulation is consistent with what has been proposed for the PS interactions at the *en* gene (KASSIS *et al.* 1991; KASSIS 1994). The PS sites in this locus are thought to function by promoting communication (positive or negative) between distant regulatory sites and the *en* promoter. A more specific model for the function of PS regions was proposed based on studies of such regions in the *ph* and the *Scr* loci (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995). Because the extent of DNA-mediated *white* repression at *ph* and *Scr* is affected by some of the Pc-G and trx-G loci, it has been suggested that multimeric protein complexes consisting of members of these gene groups associate with PS sites to control gene expression by altering chromatin structure (see above section). However, the PS regions of *pb* and *en* do not appear to be directly regulated by members of the Pc-G and trx-G loci, suggesting that a more general model for the function of PS sites, such as the one proposed here, is necessary (this report; KASSIS 1994).

Central to all models concerning the functions of PS sites is the assembly of a protein complex. At present, there is no direct evidence that protein complexes interact with the PS sites of *pb*. However, because the PS regions of *pb* contain positive or negative regulatory sequences that affect gene expression, multiple proteins are likely to be bound to them. The fact that the Pc-G and trx-G proteins interact with the PS sites at the *ph* and *Scr* loci provides the strongest evidence, to date, that protein complexes may bind to PS elements (FAUVARQUE and DURA 1993; GINDHART and KAUFMAN 1995). In addition, CHAN *et al.* (1994) found a regulatory region of the *Ubx* locus that is capable of repressing *white* in a *P*-element construct and that contains elements that form a complex with the products of the Pc-G genes.

Finally, the results presented in this report demonstrate that some PS regions can function independently of the genomic DNA that flanks the PS-containing transgene. The idea that these genomic regions may sometimes interact with transgenic PS sequences was first suggested from studies at the *en* locus (KASSIS 1994). A deletion flanking an *en* PS region appeared to abolish *white* repression that was mediated by this fragment. It was proposed that an interaction (in *cis*) between the transgenic PS elements and another PS region located in the deleted genomic sequences may be important for the repression of *white*. The 0.58-kb *pb* PS region does not appear to require flanking genomic PS sites to function, because it mediates *white* repression in all independent transformant lines. If the PS phenotype conferred by this fragment was dependent on flanking genomic PS sites, some of the 0.58+HZR transformant lines would not be expected to be PS; a certain percentage of the transgenes in these lines may not have inserted near a genomic PS region. Future discoveries of PS regions will test the validity of the proposed model for how PS sites function.

Synapsis-sensitive PS DNA-mediated effects: The homologous chromosomes of Drosophila are paired in diploid nuclei (METZ 1916; KOPCZYNSKI and MUSKAVITCH 1992; HIRAOKA *et al.* 1993), indicating that the PS regions located in each homolog (in the homozygous condition) are in close proximity to each other. This nearness may facilitate an interaction between proteins that are bound to the PS elements in each homolog, possibly resulting in cooperative or enhanced binding of these *trans*-acting factors (HAZELRIGG and PETERSON 1992). These protein-bound PS sites may interfere with the *trans*-acting regulatory factors bound to the *white* promoter causing transcription to be repressed. The precise manner in which the PS DNA mediated *white* repression occurs is not known. However, the observation of DNA-mediated pairing sensitive gene regulation is not a new one. In 1954 E. B. LEWIS described an effect known as transvection at the Bithorax complex (LEWIS 1954). Transvection describes the phenomenon of allelic complementation that is dependent on chromosome pairing. It is postulated that enhancers on one homolog affect the promoter sequences on the other homolog and influence gene regulation (GEYER *et al.* 1990). Transvection has been documented at numerous loci in Drosophila, for example, *decapentaplegic, cubitus-interruptus, brown, Notch, white, light,* 64C puff, *Sgs-4, yellow,* and *Scr* (reviewed in JUDD 1988; ASHBURNER 1989; TARTOF and HENIKOFF 1991; WU 1993). In addition, limited data exist for transvection at *pb* (T. C. KAUFMAN, unpublished observation). Two *pb* alleles behave differently when heterozygous with a *pb* null allele that allows for pairing than they do with one that disrupts pairing. Only in the latter case is a *pb* hypomorphic phenotype observed (the former is wild type), suggesting that the integrity of paired chromosomes in these genetic backgrounds is important for *pb* function. The fact that discrete *pb* regulatory regions were found to be sensitive to the pairing of chromosomes is consistent with this observation.

Is chromosomal pairing important for gene regulation? The numerous examples of transvection and PS effects suggest a functional role for pairing. Interestingly, there is evidence for somatic pairing in mammals and *trans*-inactivation in plants (GERMAN 1974; SCRABLE *et al.* 1987; LANGLOIS *et al.* 1989; NAPOLI *et al.* 1990; VAN DER KROL *et al.* 1990). However, *trans*-regulation appears not to be essential because most genes, including those within the Antennapedia complex, function properly when pairing is disrupted. Thus, the significance of *trans*-regulation may be more subtle. For example, it could provide for more efficient regulation or a

redundancy of function that is important only when the normal regulatory machinery is somehow compromised.

Synapsis-independent PS DNA-mediated effects: If protein complexes interacting with PS sites interfere with *white* transcription, one could imagine that *white* repression would occur in both heterozygous and homozygous animals, and in the latter condition, repression might be enhanced as a result of an increase in the number of proteins bound near the *white* promoter. In fact, some *pb* PS regions exhibit *white* mottling in the heterozygous condition; 67% of 9.6 + /-HZR transformants and 45% of all 2.1 lines linked to the *pb* promoter showed mottled or patterned eyes as heterozygotes. These percentages are considerably higher than the frequency of *P*-element insertion near heterochromatin, which often results in anomalous *white* expression (HAZELRIGG *et al.* 1984; LEVIS *et al.* 1985a), indicating that heterochromatic effects cannot be solely responsible for the observed *white* mosaicism in heterozygote animals. When transformants harboring the 9.6- and 2.1-kb fragments (in *P*-element vectors) were made homozygous, repression was more extreme, sometimes resulting in complete *white* inactivation. The actual significance of *white* repression in heterozygotes is not known, because other transformant lines that exhibit a strong PS phenotype do not exhibit significantly high percentages of *white* repression as heterozygotes. For example, all transformant lines of 0.58+HZR are PS (9/9), but only 8% (1/12) of the lines show mottling in heterozygous animals. Because the 0.58-kb sequence is a subregion of the 9.6-kb fragment, it is possible that multiple PS elements exist in the larger fragment and are able to "pair" in *cis* with each other, resulting in more protein complex formation and thus more *white* repression in the heterozygous transformants. According to this model, the smaller 0.58-kb PS region may have fewer sites available for *cis*-pairing; therefore, less efficient *white* repression is observed in heterozygotes. It is also possible that some of the *white* mosaicism observed in heterozygotes (*e.g.*, the 1 of 12 lines of the 0.58+HZR transformant animals) may be due to position effects from flanking genomic DNA and/or tandem *P*-element insertions, which have been shown to cause *white* repression in heterozygous animals (DORER and HENIKOFF 1994). Further molecular characterization of the PS regions of *pb* and the genomic sequences flanking the *P*-element insertions (containing these regions) will be required to test these models.

PS sites may help identify important regulatory DNA

If PS regions are DNA sequences that contain sites for protein complexes or aggregates that can function to regulate gene expression, why are there relatively few reports of this phenomenon in Drosophila? An explanation could be that the *white* minigene has only recently been used as a marker for *P*-element transformation; it has replaced the *rosy* gene, which was previously in widespread use. Because *white* is cell-autonomous (unlike *rosy*), cell to cell differences in the eye can be easily observed. In addition, two copies of the *white* minigene are readily distinguished from one copy, because the transgene lacks its normal regulatory elements that are responsible for high levels of *white* expression (LEVIS *et al.* 1985b). In contrast, doubling the dose of *rosy* does not produce a readily distinguishable increase in eye pigmentation. It is possible that the absence of the *white* regulatory elements makes this reporter gene more sensitive to the effects of PS regions. Thus, *P*-element vectors containing the *white* minigene appear to be useful reporter genes for the detection of PS regions, and as demonstrated in this study, the use of these vectors may facilitate the identification of important gene regulatory sequences.

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