Unusual Properties of Regulatory DNA From the Drosophila *engrailed* Gene: Three "Pairing-Sensitive" Sites Within a 1.6-kb Region

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ABSTRACT

We have previously shown that a 2-kb fragment of *engrailed* DNA can suppress expression of a linked marker gene, *white*, in the *P* element vector CaSpeR. This suppression is dependent on the presence of two copies of *engrailed* DNA-containing *P* elements (*P[en]*) in proximity in the Drosophila genome (either in *cis* or in *trans*). In this study, the 2-kb fragment was dissected and found to contain three fragments of DNA which could mediate *white* suppression [called "pairing-sensitive sites" (PS)]. A PS site was also identified in regulatory DNA from the Drosophila *escargot* gene. The eye colors of six different *P[en]* insertions in the *escargot* gene suggest an interaction between *P[en]*-encoded and genome-encoded PS sites. I hypothesize that *white* gene expression from *P[en]* is repressed by the formation of a protein complex which is initiated at the *engrailed* PS sites and also requires interactions with flanking genomic DNA. Genes were sought which influence the function of PS sites. Mutations in some Polycomb and trithorax group genes were found to affect the eye color from some *P[en]* insertion sites. However, different mutations affected expression from different *P[en]* insertion sites and no one mutation was found to affect expression from all *P[en]* insertion sites examined. These results suggest that *white* expression from *P[en]* is not directly regulated by members of the Polycomb and trithorax group genes, but in some cases can be influenced by them. I propose that *engrailed* PS sites normally act to promote interactions between distantly located *engrailed* regulatory sites and the *engrailed* promoter.

SPATIALLY and temporally regulated transcription relies on positive and negative *cis*-acting sequences called enhancers and silencers. At least for transcriptional enhancers, these sequences can be located 10s of kilobases away. For the *engrailed* (*en*) locus of *Drosophila melanogaster*, sequences located up to 40 kb upstream and 20 kb downstream of the promoter are thought to regulate transcription (KUNER *et al.* 1985; DREES *et al.* 1987). The problem of how such distantly located regulatory elements influence transcription is largely unsolved. One model is that proteins that bind near the promoter interact with proteins bound to the enhancer and cause a looping of intervening DNA (MULLER and SCHAFFNER 1990). This model requires the interaction of proteins bound to sites separated by large distances on a linear scale. One might imagine that the same proteins could mediate an interaction between noncontiguous pieces of DNA. We have identified a fragment of *en* regulatory DNA which behaves in a manner consistent with such an ability: first, it promotes interactions between transposons located on homologous chromosomes (the pairing-sensitive phenomenon; KASSIS *et al.* 1991) and second, it directs the insertion of *P* elements to particular regions of the Drosophila genome (KASSIS *et al.* 1992)

We previously reported that a fragment of *en* DNA had an unusual effect on a linked marker gene, *white*, in the *P* element transposon CaSpeR (KASSIS *et al.* 1991). Normally, flies homozygous for CaSpeR, having two copies of *white*, have darker eyes than heterozygotes (PIRROTTA 1988). However, when a particular fragment of *en* DNA was included in the construct, homozygous flies often had a lighter eye color than heterozygous flies. This suppression of *white* expression was dependent on the proximity of the two transposons–either in *cis* or in *trans*. Thus, this phenomenon could be classified as a transvection effect (JUDD 1988; PIRROTTA 1990). We postulated that proteins bound to the *en* fragment on one chromosome interact with proteins bound to the *en* fragment on the other chromosome, and that this protein complex interferes with *white* transcription.

Inclusion of *en* regulatory DNA in *P* element vectors (referred to as *P[en]*) resulted in selective insertion into the Drosophila genome (KASSIS *et al.* 1992). Many of the genes where selective insertion occurs are expressed in striped patterns in the embryo. We postulate that selective insertion of *P[en]* occurs when proteins bound to the *en* fragment in the *P* element vector interact with proteins bound to genomic DNA, concentrating *P[en]* in particular regions of the genome resulting in a higher frequency of insertions in those regions.

With the goal of identifying proteins that mediate these two phenomena, subsets of this *en* fragment were assayed in CaSpeR for the ability to suppress *white*

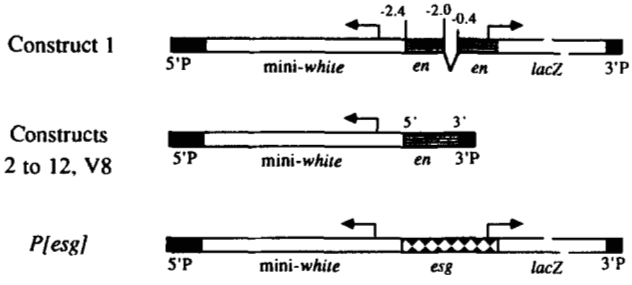

FIGURE 1.—Transposon constructs. In construct 1, *D. melanogaster en* DNA (extending from −2.4 kb to −2.0 kb and from −400 bp to +188 bp) was cloned in front of the *lacZ* gene in the vector pC4ATGβgal (THUMMEL *et al.* 1988), and then the *en-lacZ* fragment was cloned into the *P* element vector CaSpeR (PIRROTTA 1988) in the orientation shown. The arrows indicate the start site of transcription from the *en* and *white* promoters. In *P[esg]*, DNA from the *escargot* gene (*esg*) was cloned in front of *lacZ* in the same vector. The presumed start site of transcription from the *esg* promoter is indicated by an arrow. In constructs 2 through 12 and V8, *en* DNA from either *D. melanogaster* or *D. virilis* was cloned into CaSpeR in the orientation shown. See Table 1 for the exact extent of the *en* or *esg* DNA present in each construct.

expression in homozygotes. Sites that mediate this suppression of *white* are called "pairing-sensitive" (PS) sites throughout this report. Three PS sites were found within a 1.6-kb fragment of *en* DNA. In addition, one of the targets of *P[en]* insertions, the *escargot* (*esg*) gene (WHITELEY *et al.* 1992), also contains a PS site. The different eye colors of 6 *P[en]* transposon insertions near the *esg* gene suggest an interaction of transposon-encoded and flanking, genome-encoded PS sites. It is proposed that the ability of a fragment of DNA to cause pairing-sensitive suppression of the *white* gene results from its interaction with DNA binding proteins that enter into multimeric complexes which bring together distant PS sites.

MATERIALS AND METHODS

Construction of transposons: *P* element transposon constructs used in these experiments are shown in Figure 1. For construct 1, a fragment of *en* DNA that contained nucleotides −2407 to −1944 (generated by deletion of a larger fragment in M13, KASSIS *et al.* 1989) was cut out of an M13 vector with *Sph*I and *Eco*RI and cloned into construct H (KASSIS 1990) that had been partially digested with *Eco*RI and completely digested with *Sph*I. For constructs 2–12 and V8, fragments of DNA containing the nucleotides listed in Table 1 were generated using polymerase chain reaction (PCR). All primers included 18 nucleotides of *en* sequence starting with the nucleotides listed in Table 1. In addition, all 5′ primers began with the sequence GCGGAATTC and all 3′ primers began with the sequence GCGGGATCC. PCR fragments were cut with *Bam*HI and *Eco*RI and cloned into *Bam*HI, *Eco*RI cut CaSpeR (PIRROTTA 1988). For *P[esg]*, a PCR-amplified fragment from the *esg* gene (containing the nucleotides shown in Table 1) was cloned into pC4ATGβgal (THUMMEL *et al.* 1988). An *Eco*RI fragment that contained the *esg-lacZ* fragment was cut out with *Eco*RI and cloned into *Eco*RI cut CaSpeR. The orientation of the insertion was determined by restriction enzyme analysis.

Generation of transgenic lines: Injections were done by standard procedures (SPRADLING 1986) into a homozygous *Df(1)w67c2, y* strain (LINDSLEY and ZIMM 1992) using 300 ng/μl of *P* element construct and 150 ng/μl of p25.7WC (KARESS and RUBIN 1984). For construct 6, only transgenic line *6-1C* was obtained by injection. Insertion *6-1C* was mobilized using the endogenous transposase insertion *P[ry*$^+$*Δ2-3]*99B (ROBERTSON *et al.* 1988) and line *6-6B* was selected because of a change in eye color. Line *6-6B*, which maps to the second chromosome, was treated with *P[ry*$^+$*Δ2-3]*99B with the intention of selecting insertions that had moved off the second chromosome. During this experiment, many flies with ring-patterned eye colors were observed. Some of these flies were saved and are represented by lines *6-3A* and *6-2A* in Table 2. All other construct 6 lines were selected as transpositions off the starting *6-6B* chromosome. Lines *10-5* and *10-7*; *12-8*, *12-10* and *12-6*; *esgP1* and *esg1-8* were generated by mobilization of *10-3*, *12-1C* and *esg-1*, respectively, with *P[ry*$^+$*Δ2-3]*99B. All other lines were obtained by injection.

Structure of the double transposons: Three lines that had double transposon insertions were used in these studies. The structures are shown in Figure 2. Line *6-6B* was selected as a change in eye color by transposition of a transposon located on the third chromosome. Line *G6-28* was selected as a white-eyed derivative of line *G6* after treatment with *P[ry*$^+$*Δ2-3]*99B. Line *B14* was derived from line *B15* by selection for a change in eye color (see KASSIS *et al.* 1991). The structures of these complex transposons were determined by Southern analysis and confirmed by PCR using multiple primers to the *white* gene, the *en* gene and the *P* element ends. Multiple primer combinations confirmed the structures shown in Figure 2A. Examples of confirmatory PCR products are shown in Figure 2B. For the discussion below, the *white* base pair numbers are taken from the CaSpeR sequence (PIRROTTA 1988); a 5′ *white* primer indicates a primer which extends in the direction of *white* transcription, a 3′ *white* primer extends in the other orientation. In lane 1, *6-6B* DNA was amplified using a 5′ *white* primer at base pair 3563 and a primer that hybridized 53 bp into the 5′ *P* element end, extending toward the 5′ *P* element end. A product of ~300 bp was obtained, suggesting that the second transposon was inserted near base pair ~3800 in the CaSpeR sequence. In lane 2, *6-6B* DNA was amplified using a primer pointing toward the 3′ *P* element end and a 3′ *white* primer at 3844. A product of ~150 bp was obtained, including 67 bp of *P* element end and ~80 bp of *white* sequences, suggesting an insertion site of ~3760 (the end of the *white* coding region is at 3394). In *G6-28*, the second element is inserted at base pair 30 within the 5′ *P* element end (determined by sequence analysis of an inverse PCR product, data not shown). Lane 3 shows the product obtained using a 5′ *white* primer at base pair 3563 (where bp 4782 is the last base of the element) and a 3′ *P* element primer pointing toward the 3′ *P* element end (67 bp before the end). The size of the product (~1350 bp) suggests that both *P* elements are intact and Southern analysis confirmed this. Lane 4 (Figure 2B) shows the product amplified from *B14* DNA using a 5′ *white* primer at base pair 3000 and one 67 bp from the 3′ *P* element end (pointing toward the 3′ end). The size of the product (~480 bp) suggests that the second *P* element is broken very near the end of the *white* coding region, which is at base pair 3394.

Mapping of transposon insertions: The chromosomal assignments of insertions were first determined by segregation tests, using balancer stocks for the second and the third chromosomes. [Second chromosome balancer stock is *w; nub b Sco lt stw*^{3}*/SM6a; Dp(2;Y)A161, B*^{s}; third chromosome balancer stock is *y w; TM3, Ser/Sb.*] The insertion sites were then localized on the salivary chromosome map by *in situ* hybridization of digoxygenin-labeled

TABLE 1

Extent of *en* fragments present in constructs and summary of results

Construct	5′ Primer[a]	3′ Primer[b]	Fragment size (bp)	Pairing sensitive[c]	Patterned eye[d]
1	−2407	−1944	463	0/5 (0)	0/5 (0)
2	−1525	−400	1125	3/3 (100)	1/4 (25)
3	−1184	−400	784	4/7 (57)	3/8 (37)
4	−1944	−1166	778	7/9 (77)	1/13 (8)
5	−1944	−1503	441	3/5 (60)	1/5 (20)
6[e]	−1184	−944	240	1/4 (25)	2/6 (33)
7	−864	−400	293	4/4 (100)	0/8 (0)
8	−576	−400	176	6/7 (86)	1/7 (14)
V8	−758	−572	186	1/1 (100)	0/1 (0)
10	−693	−559	134	0/6 (0)	0/6 (0)
12	−454	−400	54	0/5 (0)	0/5 (0)
esg	1	985	985	4/4 (100)	0/6 (0)
B, E, F, G[f]	−2407	+188	2595	14/18 (78)	8/22 (36)
H & I[f]	−400	+188	588	0/11 (0)	2/16 (12)

[a] Number of the first nucleotide of the fragment from the *D. melanogaster en* sequence (1–8, 10 and 12; KASSIS *et al.* 1989), the *D. virilis en* sequence (V8; KASSIS *et al.* 1989) and the *D. melanogaster esg* sequence (WHITELEY *et al.* 1992).

[b] Number of the last nucleotide of the fragment.

[c] Number of lines where the eye color of homozygotes is lighter than or indistinguishable from the eye color of heterozygotes/total number of homozygote viable lines. Numbers in parentheses are percent.

[d] Number of lines with a patterned eye color/total number of lines. Numbers in parentheses are percent.

[e] Line *6–6B* has a double transposon and was not included in this table. Lines *6–2A* and *6–3A* were inserted at the same site and were not both included as lines having patterned eyes.

[f] These data are taken from KASSIS *et al.* (1991). Construct G has a deletion which extends from −854 to −950. Some of these lines also contain the *D. virilis* or *D. melanogaster en* intron.

white DNA (a *Sad* fragment extending from the polylinker in the CaSpeR vector to the *SacI* site within the *white* gene). Digoxygenin labeling and detection were done with the Genius kit (Boehinger Mannheim). Preparation of polytene chromosomes and hybridization followed the methods of ENGELS *et al.* (1986). The insertions at *esg* were mapped using PCR as described in WHITELEY *et al.* 1992.

Examination of eye color: In the discussion that follows all genotypes are homozygous or hemizygous null for their endogenous *white* genes. All genes and mutations are described in LINDSLEY and ZIMM (1992) except the following: $ash1^{B1}$, trx^{B19}, trx^{B25} and kto^{3} (unpublished alleles provided by JAMES KENNISON). Psc^{1443} is the same as $l(2)49Ea^{2}$ (LINDSLEY and ZIMM 1992). In all experiments, the eye colors of homozygotes were compared with the eye colors of similar age and sex heterozygotes.

To test the effects of Polycomb and trithorax group mutations on the expression of the *white* gene in the transposons, mutations in flies heterozygous for both the transposon and the mutation were initially tested. Females that contained the insertion were crossed to males heterozygous for the mutation and a marked balancer. Since most of the mutation stocks contained a wild-type *white* gene, only the eye color of males could be examined. The eye color of unmarked males was compared with those that contained the marked balancer. Flies were scored as different only if they could be sorted into two groups on the basis of eye color alone without identification of the genotype. In initial experiments, mutations in Polycomb and trithorax group genes were found to have no effect on the eye color of heterozygous single transposon flies with insertions at three different locations (*E7* (78E), *F4* (48A) and *G6* (35C)). Chromosomes containing the following mutations were tested: Pcl^{10}, trx^{E2}, kis^{1}, *Df(3L)kto2*, *e(Pc)* (with *E7*, *F4* and *G6*); kto^{1}, urd^{2}, brm^{2}, *Df(2R)Ba*, vtd^{5}, sls^{1}, osa^{1}, Pc^{4}, *Df(2L)JK12*, mor^{1}, dev^{1} (with *E7* and *G6*); $l(3)87Ca^{16}$, Asx^{1}, skd^{2} (tested with line *E7* only).

For examination of the effect of the *trithorax* (*trx*) mutation on the eye color of flies homozygous for a transposon, lines with transposons on the second chromosome that had at least some color as a homozygote were chosen, since *trx* mutations lead to a decrease in eye color in *B14* heterozygotes. Homozygous insert lines were crossed to *y w; TM3, Ser/Sb. TM3,Ser* daughters carrying the transposon (w^{+}) were crossed to *cn* en^{1}*/SM6a; ru h th st* trx^{E2}*ca/TM6C, Sb* males. Transposon (w^{+})*/SM6a; ru h th st* trx^{E2}*ca/TM3, Ser* males were crossed to homozygous transposon-containing females and the eye color of non-*SM6a, Ser* and non-*Ser* flies were compared.

β-Galactosidase assays: Homozygous larvae were collected from homozygous transgenic parents. Heterozygous larvae were collected from homozygous transgenic males crossed to *Df(1)w67c2, y* females. Larvae were dissected in phosphate-buffered saline (PBS) by removing the anterior one-half and inverting, and then they were transferred to PBS on ice. To ensure that discs of different genotypes were handled equivalently, the gut was removed from larvae of one genotype and left on the other. Five larvae from each genotype were put together in a single Eppendorf tube. PBS was removed and larvae were fixed with 1% glutaraldehyde in PBS for 15 min at room temperature. Larvae were then rinsed two times with X-gal buffer (1 mM MgCl, 150 mM NaCl, 10 mM $NaPO_4$, pH 7.2), and incubated for 5 min in X-gal buffer before applying the staining solution (1.6% X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, in X-gal buffer). Larvae were incubated for 5 h at room temperature in staining solution, rinsed with PBS, and post-fixed in 1% glutaraldehyde in PBS for 15 min prior to dissection in PBS. Discs were mounted in 80% glycerol and examined using Nomarski optics.

For β-galactosidase activity assays, three larvae per group were homogenized in 500 μl of 50 mM potassium phosphate, 1 mM magnesium chloride (pH 8.0) on ice, and then centrifuged at 12,000 rpm for 15 min at 4°. A 50-μl aliquot of supernatant was incubated with 5 mM chlorophenol red-β-D-galactopyranoside (CPRG, Boehinger Mannheim) at room temperature for 30 min to 2 hr, and the OD_{574} of the solution was determined spectrophotometrically (SIMON and LIS 1987).

TABLE 2

Transgenic lines

Name[a]	Location	Homozygote/ heterozygote[b]	Patterned[c]	Name[a]	Location	Homozygote/ heterozygote[b]	Patterned[c]
1–8	2F	D	No	*6–2B*	1EF	D	No
1–15	2F	D	No	*6–1A*	12C	D	No
1–18	5B	D	No	*6–6B*[d]	35C and 35C	D	No
1–29	58EF	D	No	*6–3A*	35C	D	Yes
1–26	67B	D	No	*6–2A*	35C	L	Yes
2–20A	21B	Lethal	No	*6–16C*	48A	On CyO	Yes
2–20B	26F	I	No	*6–1C*	100F	D	No
2–4	52A	L	No	*7–8B*	29D	L	No
2–17	55C-F	L	Yes	*7–9B*	85D	I	No
3–11D	35C	L	Yes	*7–8A*	87AB	L	No
3–10	66A	L	No	*7–11*	88AB	L	No
3–3	67AB	L	No	*8–4B*	3A	L	No
3–9	85D	D	Yes	*8–10B*	19A	I	No
3–13	89AB	D	No	*8–10A*	28A	L	No
3–15	89C	L	Yes	*8–10C*	53F	L	No
3–11A	94B	D	No	*8–4A*	71A	L	No
3–11E	98F	Lethal	No	*8–6*	93A	L	No
4–15A	1EF	I	No	*8–4C*	95B	D	Yes
4–1A	2C	I	No	*V8–2*	On *2*	L	No
4–19B	7A-C	I	No	*10–3*	25D	D	No
4–12E	48A	Lethal	No	*10–4*	On *3*	D	No
4–12A	52DE	L	Yes	*10–7*	61D	D	No
4–7B	55C	Lethal	No	*10–5*	65F	D	No
4–16B	58F	D	No	*10–2*	68D	D	No
4–3A[d]	59AB and 50CD	Lethal	No	*10–1*	98B	D	No
4–7A	61B	D	No	*12–8*	On the *X*	D	No
4–16A[d]	75F and 88C	D	No	*12–1C*	32A	D	No
4–16C	85F	I	No	*12–1E*	85D	D	No
4–12C	92B	L	No	*12–10*	64A-65A	D	No
4–15B	98DE	I	No	*12–6*	83A	D	No
5–18	28BC	I	No	*esg-1*	49D	L	No
5–7F	31F	L	No	*esg-P1*	On *3*	L	No
5–7A	40A	D	Yes	*esg-5*	69AB	Lethal	No
5–7C	58DE	L	No	*esg-2*	91B	L	No
5–13	88CD	D	No	*esg-18*	96D-F	L	No

[a] Construct name-line number.

[b] D, eye color of homozygote darker than eye color of heterozygote; L, eye color of homozygote lighter than eye color of heterozygote; I, eye color of homozygote indistinguishable from eye color of heterozygote; lethal, homozygous insertion is lethal and, therefore, the eye color of homozygotes could not be examined.

[c] Transgenic lines in which the eye color was not homogeneous, but where one portion of the eye regularly was more highly pigmented were called "patterned." Patterning was evident in homozygotes and/or heterozygotes, and was a stable and consistent characteristic of each line.

[d] These lines were not included in the summaries because of the presence of two transposons.

Care was taken to ensure that the measured activities were within the linear range of the assay.

RESULTS

We have previously shown that a 2-kb fragment of *en* DNA (extending from −2407 to −400) exhibited pairing-sensitive suppression of the *white* gene present in the *P* element vector CaSpeR (KASSIS *et al.* 1991). To further localize the DNA responsible for this phenomenon, this fragment was subdivided and tested for effects on the expression of the *white* gene of CaSpeR in transgenic flies. The nucleotides present in each fragment tested are listed in Table 1. The configuration of the transposon for each fragment is shown in Figure 1. A listing of the transgenic lines generated is shown in Table 2 and the results are summarized in Figure 3. In construct 1, two *en* fragments are present, one from −2.4 kb to −2.0 kb and the other extending from −0.4 kb to +188 bp. We have previously shown that the fragment extending from −0.4 kb to +188 bp does not mediate pairing-sensitive suppression of the *white* gene (construct H and I, Table 1, results from KASSIS *et al.* 1991). The *en* fragment extending from −2.4 kb to −2 kb also does not contain a PS site since no transgenic line containing Construct 1 mediated pairing-sensitive suppression of the *white* gene. Therefore, I have concentrated on *en* sequences extending from −2.0 kb to −0.4 kb.

At least three pairing-sensitive sites occur within a 1.6-kb fragment of *en* DNA: Fragments 2 through 5 were the first tested for the presence of PS sites (see Table 1 and Figure 3). At least two sites were identified by these experiments, a 441-bp fragment in construct 5, and a 784-bp fragment in construct 3. Sequence comparisons of these two fragments detect a region of 25 bp with 80% identity between the two fragments (from −646 to −621 in construct

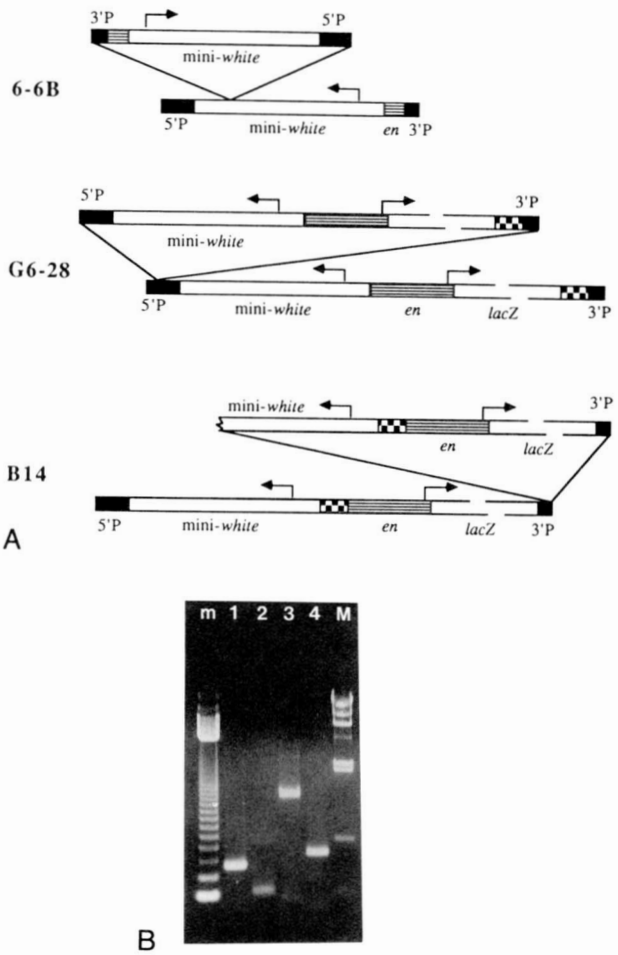

FIGURE 2.—Double transposon lines used in these studies. (A) The structure of each double transposon is shown. *G6-28* has two complete construct G transposons, one inserted 30 bp from the 5′ end of the other. *6-6B* has two construct 6 transposons, one inserted approximately 400 bp 3′ to the stop codon of the *white* gene of the other. Line *B14* has one complete and one incomplete construct B transposon. The incomplete *white* gene ends in the vicinity of the stop codon of the *white* gene. Constructs G and B have the *D. virilis* and *D. melanogaster en* intron 1, respectively (shown as the checkered box). (B) PCR products obtained confirming the structures shown in (A). Lane 1, *6-6B* DNA amplified with a primer from the 5′ *P* element end and a 5′ *white* primer at 3563. Lane 2, *6-6B* DNA amplified with a primer from the 3′ *P* element end and a 3′ white primer at 3844. Lane 3, *G6-28* DNA amplified with a 5′ white primer at 3563 and a primer from the 3′ *P* element end. Lane 4, *B14* DNA amplified with a 5′ white primer at 3000 and a primer from the 3′ *P* element end. m, 123-bp ladder; M, λ *Hin*dIII.

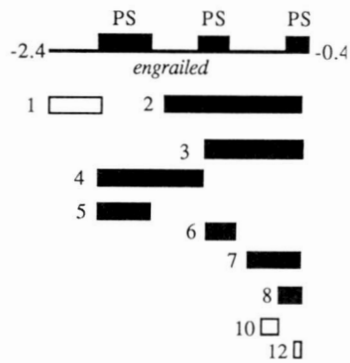

FIGURE 3.—Pairing-sensitive sites (PS) present in *D. melanogaster engrailed* DNA. The top line represents the extent of *en* DNA tested in this paper and the location of the PS sites determined from the constructs (shown below). Unfilled boxes indicate that pairing-sensitive suppression of *white* did not occur in transgenic lines obtained from that construct. Filled boxes indicate that pairing-sensitive suppression of *white* was found in transgenic lines obtained from that construct.

3 and from −1641 to −1616 in construct 5). However, this sequence is neither required nor sufficient to mediate pairing-sensitive suppression of *white*; construct 10, which contains it, did not contain a PS site, and constructs 6 and 8, which lack it, do contain PS sites.

Fragment 3 was further dissected and found to have two PS sites: one strong site present in fragment 8 and a weak site present in fragment 6 (see Tables 1 and 2). Six of seven transgenic lines that contain fragment 8 exhibited pairing-sensitive suppression of the *white* gene suggesting that fragment 8 confers PS relatively independent of chromosomal insertion site. In contrast, only one of six transgenic lines that contain fragment 6 exhibited pairing-sensitive suppression of *white* suggesting that this suppression may be more easily overcome by interactions with flanking DNA. Sequence comparisons between fragments 6, 8 and 5 did not reveal any similarities at a level of 8/10 matches. There are a number of very short regions of sequence identity between the three fragments; the significance of these is not clear.

We had previously shown that PS sites are also present within homologous *Drosophila virilis en* DNA (KASSIS *et al.* 1991). Here a 186-bp fragment of *D. virilis* DNA that is homologous to the region present in construct 8 was tested (V8). Construct V8 did contain a PS site; thus, this site is a conserved feature of *en* DNA. Sequence comparison between fragment V8 and fragment 8 reveals three regions of near identity, a 16/17 base pair match (from *Drosophila melanogaster* nucleotide −547 through −530; nucleotide numbers are from KASSIS *et al.* 1989), a 20/23 base pair match (from *D. melanogaster* nucleotide −481 through −458), and a 27/31 base pair match (from *D. melanogaster* nucleotide −431 through −400). The longest region of identity was present in construct 12. No transgenic lines derived from construct 12 exhibited pairing-sensitive suppression of *white*.

A PS site is located within 1 kb upstream of the *esg* ATG: The *esg* gene is a target for directed *P[en]* insertions (WHITELEY *et al.* 1992; see below). Since the models I have proposed for pairing-sensitive suppression and for selective insertion of *P[en]* both rely on protein-protein interactions, I decided to test whether *esg* DNA causes selective insertion of transposons and whether it contains a PS site. A fragment of 985 bp of *esg* DNA (base

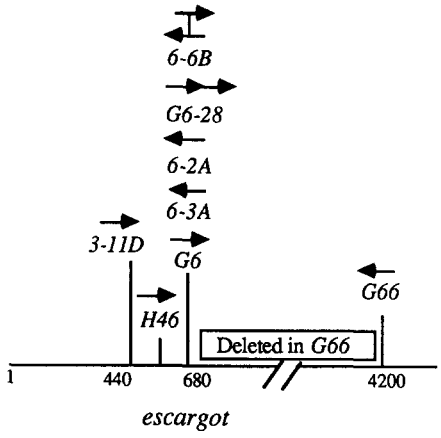

FIGURE 4.—Transposons inserted at *escargot*. The horizontal line represents *esg* DNA with the base pair number indicated below (WHITELEY *et al.* 1992). The location of the transposon insertion in each transgenic line is indicated by a vertical line. The orientation of the transposon present in each line is indicated by the arrow (5′ pointing to 3′). Lines *G6-28* and *6-6B* both have two *P* elements whose orientation is shown (see Figure 2). The 3.5-kb deletion of *esg* sequences present in line *G66B* is shown. Each line with a single transposon is listed below with the number of PS sites and the eye color of transgenic flies.

Line	*engrailed* sequences	# of PS sites	heterozygous eye color	homozygous eye color
G6	-2407 to +188	3	light orange ring	white
3-11D	-1184 to -400	2	dark orange ring	light yellow ring
6-3A	-1184 to -944	1	light orange ring	orange ring
6-2A	-1184 to -944	1	dark orange ring	white
G66	-2407 to +188	3	light red, no ring	lethal
H46	-400 to +188	0	orange, no ring	dark red

pair 1000 is the beginning of the *esg* coding region) was cloned into a *P* element vector that contains the *white* gene as a marker and *lacZ* as a reporter gene (Figure 1, *P[esg]*). While not enough lines have been established to determine whether *P[esg]* is selectively inserted in the genome, *P[esg]* contains at least one PS site. In all four homozygous viable *P[esg]* lines obtained, the eye color of homozygotes is much lighter than that of heterozygotes (Tables 1 and 2). Like the *en* fragments that contain PS sites, this *esg* fragment has no tissue-specific enhancer activity.

Mapping of insertions to escargot: The locations of 8 *P[en]* insertions at *esg* and the *en* fragment present in each single transposon line are shown in Figure 4. The data on the insertion sites of lines *H46*, *G6* and *G66* are from WHITELEY *et al.* (1992). Four of the insertions were independent events (*G6*, *H46*, *3-11D* and *6-6B*). Lines *G66* and *G6-28* were derived from line *G6*, and lines *6-2A* and *6-3A* were derived from line *6-6B*, by treatment with *P* element transposase. In line *G66* (which was selected by a change in eye color), the *G6* transposon flipped orientation and 3.5 kb of *esg* sequences were deleted (WHITELEY *et al.* 1992). In line *G6-28* (which was selected as a white-eyed derivative of line *G6*), transposon G duplicated creating the structure shown in Figure 2.

Line *3-11D* has a patterned eye color similar to line *G6*, which was known to be inserted in *esg* (WHITELEY *et al.* 1992). A suppression of eye color was found to occur in *3-11D/G6 trans*-heterozygotes (Table 3), showing that the transposons present in these two lines interact. By *in situ* hybridization to polytene chromosomes, the insertion in line *3-11D* is at 35C, which is the

TABLE 3

Interactions between *P* element constructs inserted at *escargot*

Line	*G6*	*3-11D*	*6-3A*	*H46*	*6-2A*	*G66*
G6	L	L	D/L	D	I/L	D
3-11D	L	L	I/D	D	L	D
6-3A	L/D	D/I	D	D	D/I	D
H46	D	D	D	D	D	D
6-2A	L/I	L	I/D	D	L	D
G66	D	D	D	D	D	Lethal

L, lighter than either parental heterozygote; D, darker than either parental heterozygote; D (L, I)/L(D, I), darker (lighter, indistinguishable) than the parental heterozygote listed in the left column/ lighter (darker, indistinguishable) than the parental heterozygote listed on top.

location of *esg*. Finally, a PCR product was obtained using primers from *esg* DNA and *P* element ends on *3-11D* DNA (Figure 5, lane 1), localizing the insertion in *3-11D* to *esg* base pair 440 in the orientation shown in Figure 4.

Line *6-6B* has a double transposon of the structure shown in Figure 2 inserted at *esg*. Line *6-6B* has uniformly colored orange eyes that are darker orange in homozygotes. Treatment of line *6-6B* with *P* element transposase generated lines *6-3A* and *6-2A*. Both these lines were selected because the eyes are colored in a ring-pattern similar to the pattern in lines *G6* and *3-11D*. In line *6-3A*, the internal transposon of line *6-6B* was lost, leaving a single transposon. This was demonstrated by inverse PCR and confirmed by Southern blot analysis (data not shown). A single transposon is also present in line *6-2A*; however, the 5′ *P* element end has been deleted in this line (Figure 5). PCR amplification using primers to *esg* DNA and *P* element sequences showed that the transposons present in lines *6-6B*, *6-3A*

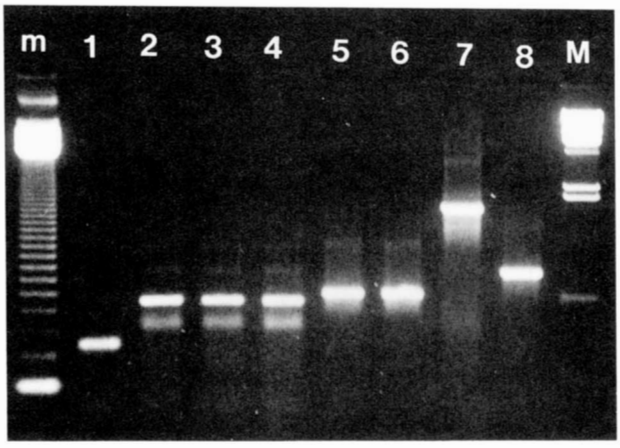

FIGURE 5.—PCR on transposons at *escargot*. Lanes 1–4, PCR using a primer starting at base pair 224 in the *esg* sequence and a *P* element primer from either the 5′ (for line *3-11D*, lane 1) or the 3′ end (for lines *6-6B*, *6-3A* and *6-2A*, lanes 2–4). For lanes 5 and 6, a 5′ *P* element primer and a primer beginning at base pair 1185 of the *esg* sequence was used on *6-6B* (lane 5) and *6-3A* (lane 6). No band was seen with this primer combination on DNA from line *6-2A* (not shown). Lanes 7 and 8, PCR on line *6-3A* (lane 7) and *6-2A* (lane 8) using a primer from within the *white* gene of CaSpeR and a *esg* primer at 1185. Line *6-2A* has a 1.2-kb deletion of *white* and *P* element end sequences and therefore gives a smaller product than that from *6-3A*. m, 123-bp ladder; M, λ *Hin*dIII.

and *6-2A* are located at nucleotide 680 in the *esg* sequence in the orientations shown in Figure 4. PCR amplifications using primers from nucleotides 400–417 of *esg* and the end of the 3′ *P* element yield identical bands for lines *6-6B*, *6-3A* and *6-2A*. PCR amplifications with primers from nucleotides 780–765 of *esg* and the end of the 5′ *P* element yield identical bands for lines *6-6B* and *6-3A*, but no band for line *6-2A* showing that the 5′ *P* element end has been deleted in line *6-2A*. Using the same *esg* primer in combination with a primer starting at base pair 3563 of the *white* gene, a band of ~1900 bp was amplified from line *6-3A* DNA, and a band of ~800 bp was amplified from line *6-2A* DNA. This showed that about 1100 bases (either *esg* or *P* element sequences or both) have been deleted in line *6-2A*. By using additional primers to *esg* and CaSpeR sequences, it was established that not more than 20 nucleotides of *esg* DNA had been deleted and that the deletion of CaSpeR sequences occurred between base pairs 3855 and 4000 (the end of the *white* coding region is at nucleotide 3394). This showed that some noncoding *white* sequences and the entire 5′ *P* element end are deleted in line *6-2A*.

Suppression of white in homozygotes depends on both genomic and transposon-encoded sequences: Constructs 3 and G both contain PS sites, and homozygous *3-11D* and *G6* flies have lighter eyes than heterozygotes (Table 3 and Figure 4). The transposon in line *6-3A* is in the opposite orientation to the transposons in *H46*, *3-11D*, and *G6*. The transposon in line *6-3A* can interact with the transposons in lines *3-11D* and *G6* to give *trans*-heterozygotes with eye colors lighter than or indistinguishable from a parental heterozygote (Table 3). This suggested that construct 6 contains a PS site. Despite this, homozygous *6-3A* flies have a darker eye color than heterozygous *6-3A* flies. In contrast, *6-2A* homozygous flies have a lighter eye color than heterozygous *6-2A* flies. Since line *6-2A* contains a 1100 base pair deletion of the 5′ end of the transposon, something within the deleted sequences may inhibit the suppression of the *white* gene in *6-3A* homozygotes.

Trans-heterozygotes made with lines *H46* and *G66* always had a darker eye color than either parental heterozygote (Table 3). The transposon present in line *H46* contains no PS site, and does not interact with the other transposons. Since *G66* also does not interact with other transposons, I suggest that the deletion of 3.5 kb of *esg* sequences in line *G66* includes a genomic PS site and that this site is necessary to obtain suppression of the *white* gene. Alternatively, deletion of the *esg* DNA may have introduced genomic DNA into the region which interferes with the PS phenomenon.

Insertions at esg suggest that a patterned eye color results from an interaction between genomic and transposon sequences: The ring-shaped patterned eye colors seen in heterozygotes of several of the transposons inserted at *esg* seem to result from an interaction between PS sites present within the transposon and in nearby genomic DNA. Four lines (*G6*, *3-11D*, *6-2A* and *6-3A*) have eyes colored in a ring pattern (the outermost part of the eye is more darkly colored than the innermost). All of these lines contain transposons with PS sites and no deletions of flanking *esg* sequences. In two other lines (*H46* and *G66*), the eyes are not colored in a ring-pattern but are evenly colored. The transposon in line *H46* does not contain a PS site. The transposon in line *G66* contains three PS sites; however, 3.5 kb of *esg* sequences have been deleted in this line, including 250 bp of the fragment included in *P[esg]*. I suggest that the PS site present at *esg* has been deleted in line *G66* and that an interaction between genomic and transposon-encoded PS sites (in *cis*) leads to the ring-patterned eye colors.

Some Polycomb and trithorax group genes affect eye colors in position-dependent manners: In a previous study we showed that mutations of *zeste*, a gene which is known to influence transvection (WU and GOLDBERG 1989; PIRROTTA 1990, 1991), had no effect on pairing-sensitive suppression of *white* in *P[en]* transposons at two chromosomal locations (line *G6* at 35C and line *E7* at 78E; KASSIS *et al.* 1991); therefore effects of other possible modifiers were examined. Mutations that suppress or enhance the action of *zeste* identify members of the Polycomb group of genes (WU *et al.* 1989), which function to repress transcription of homeotic genes (PARO 1990). Another group of genes, the trithorax

group, antagonize the action of Polycomb group genes (KENNISON 1993). I have tested the effects of several Polycomb and trithorax group mutations on *white* expression from *P[en]* transposons.

Most Polycomb and trithorax group mutations are homozygous lethal, and thus the phenotype of flies homozygous for these mutations could not be examined. However, mutation of one copy of some Polycomb and trithorax group genes leads to loss-of-function phenotypes (KENNISON and TAMKUN 1988); therefore, the effects of heterozygous mutations on the expression of the transposon-encoded *white* gene were examined. Because I hypothesize that the eye colors of *P[en]* flies result from interactions between PS sites in the transposon with nearby genomic PS sites (see above), I first examined the effects of Polycomb and trithorax group mutations on expression in flies heterozygous for transposon. Three transposon lines were examined: *G6* (an insertion at *esg* in 35C, see above); *F4* (an insertion at *en* in 48A) and *E7* (an insertion in 78E). No effects on eye color were observed with any of the mutations tested (see MATERIALS AND METHODS).

Line *B14* contains a complex transposon containing one complete *P[en]* and a second, incomplete *P[en]* (see Figure 2). We have previously shown that these two transposons interact in *cis* to suppress *white* expression (KASSIS *et al.* 1991). Therefore, this line was used to assay the effects of Polycomb and trithorax group mutations on pairing-sensitive suppression. Flies heterozygous for the B14 transposon and heterozygous for either a marked balancer or for a chromosome containing a Polycomb or trithorax group mutation were compared. Five alleles of *trithorax* (*trx*) and two alleles of *Polycomb-like* (*Pcl*) lighten or darken the eye color, respectively. Although some mutations in other genes modified the eye color of *B14* heterozygotes, these effects were allele-specific (see Table 4). Since only effects of entire mutant chromosomes were examined in these studies, the allele-specific effects could be due to other mutations on the mutant chromosomes. No attempt was made to map the interacting-mutation because of the failure of the mutant chromosomes to affect multiple transposon insertions (see below). To determine which mutations modify *white* expression from multiple insertion sites, I tested the same mutations with line *G6-28* (data not shown; for structure, see Figure 2). Because *G6-28* heterozygous flies have white eyes, only mutations that darken the eye color were expected to modify expression. As expected, *trx* mutations (which lighten the eye color of *B14* heterozygotes) do not affect the white eye color of *G6-28* heterozygotes. Only the $Su(z)2)^1$ mutation affected the eye color of *G6-28* flies; *G6-28*/$Su(z)2^1$ *trans*-heterozygous flies have a light orange eye easily distinguishable from the white eyes of *G6-28* heterozygotes. *Pcl* mutations, which darken the eye color of *B14* heterozygotes, do not affect the eye color of *G6-28* heterozygotes.

TABLE 4

Effects of mutations in the Polycomb and trithorax group genes on the eye color of *B14* heterozygotes

Mutation	Results
Mutations that had strong effects	
Pcl^{10}	D
Pcl^{11}	D
e(Pc)	L
trx^{E2}	L
trx^{B19}	L
trx^{B25}	L
$Su(z)2^1$	D
Psc^{1433}	L
Mutations which had weak effects	
trx^{E8}	SL
trx^{E3}	SL
Df(2R)Ba	SL
Df(3L)kto2	SL
kis^1	SL
skd^2	SD
$E(z)^{60}$	SD
No effect	
z^1, z^a	
urd^2, sls^1, mor^1, $l(3)87Ca^{16}$, *Df(2L)JK12*, brm^2, kis^1, vtd^3, skd^1, Ba^B, kto^3, osa^1, $ash1^{B1}$, kis^2, osa^2, dev^1, kto^1, dev^2	
Asx^1, Pc^3, Pc^4, sxc^3, esc^2, esc^{21}, Psc^{1445}, Psc^1, $E(z)^1$	

D, eye color of *B14* heterozygotes was darker in the presence of the mutation; L, eye color of *B14* heterozygotes was lighter in the presence of the mutation. SL, eye color of *B14* heterozygotes was slightly lighter in the presence of the mutation. SD, eye color of *B14* heterozygotes was slightly lighter in the presence of the mutation.

The affect of a strong *trx* mutation (trx^{E2}) on the expression of the *white* gene in flies homozygous for single *P[en]* transposons was examined. Since *trx* mutations lighten the eye color of *B14* heterozygotes, only transposon lines that had colored eyes as homozygotes were tested. Flies with several different constructs were tested, and as shown in Table 5, trx^{E2} lightened the eye color of only two of eleven *P[en]* insertions. These two insertions (*G63* and *5-7A*) contained different constructs. Further, in these two lines, trx^{E2} lightens the eye colors of heterozygotes as well as homozygotes. This suggested that the position of insertion in the genome might determine whether a particular insertion would respond to a particular mutation. Therefore, I tested $E(z)^1$, $E(z)^{60}$, and all Polycomb and trithorax group mutations that strongly affected the eye color of *B14* flies for effects on four construct 5 insertions (Table 6). The mutation $E(z)^1$ affected the expression from three of four insertion sites, while the mutations Psc^{1443}, $E(z)^{60}$, Pcl^{11} and trx^{E2} affected only the insertion in line *5-7A*. The three remaining mutations that affected the *B14* eye color had no effect on the eye colors of any of the four insertions. Line *H16* contains an insertion of construct H, which does not have a PS site (Table 1). The transposon in line *H16* is inserted in 40A, near the site of insertion in line *5-7A* (data not shown). Although the transposon in *H16* does not contain a PS site, the two Polycomb group mutations [$E(z)^1$ and Psc^{1433}] that most strongly modified

TABLE 5

The effect of trx^{E2} on eye color is dependent on the site of insertion in the genome

Line	*P[en]/+; trx^{E2}/+*	*P[en]/P[en]; trx^{E2}/+*
F4 (48A)[a]	NE	NE
G63 (24E)	SL	L
G6 (35C)	NE	ND
3-11D (35C)	NE	NE
4-16B (58F)	NE	NE
4-12A (52DE)	NE	NE
5-7A (40A)	L	L
5-7C (58DE)	NE	NE
5-7F (31F)	NE	NE
5-18 (28BC)	NE	NE
8-10A (28A)	NE	NE

NE, trx^{E2} had no effect on the eye color; SL, eye color was slightly lighter in the presence of trx^{E2}; L, eye color was much lighter in the presence of trx^{E2}; ND, not done.

[a] (Chromosomal insertion site).

TABLE 6

Effects of Polycomb and trithorax group mutations on the eye colors of transgenic flies that are heterozygous for construct 5

	$E(z)^{60}$	$E(z)^{1}$	Pcl^{11}	trx^{E2}	Psc^{1443}	$Su(z)2^{1}$ $e(Pc)$ Pcl^{10}
5-7A (40A)[a]	SD	L[b]	SD	L	L[b]	NE
5-7C (58DE)	NE	SL	NE	NE	NE	NE
5-7F (31F)	NE	SL	NE	NE	NE	NE
5-18 (28BC)	NE	NE	NE	NE	NE	NE
H16A (40A)	NE	SL	NE	NE	L	NE

L, lighter; SD, slightly darker; SL, slightly lighter; NE, no effect.

[a] (Chromosomal insertion site).

[b] Dramatic effect. *5-7A*; $E(z)^{1}$ and *5-7A/Psc^{1443} trans*-heterozygotes have white eyes.

the eye color of *5-7A* heterozygotes also modified the eye color of *H16* heterozygotes. These results suggest that the effects of trithorax and Polycomb group mutations on *white* expression from *P[en]* transposons are dependent on the position of insertion in the genome, and thus these gene products do not act directly through the transposon-encoded PS sites.

***lacZ* expression from the *en* promoter is controlled in a position-dependent manner:** The *en* fragment extending from −2.4 kb to +188 bp does not direct expression in a tissue or stage-specific manner, but contains promoter activity which can be activated by nearby genomic enhancers (KASSIS 1990; KASSIS *et al.* 1992). In many of the *P[en]* constructions, *lacZ* is expressed from this *en* promoter-containing fragment (see construct 1 for the orientation of the *en* and *white* promoters, also see KASSIS *et al.* 1991). I sought to examine whether the *en* promoter, like the *white* promoter, was repressed in *P[en]* lines. In the first experiment, β-galactosidase expression in imaginal discs from *B14* heterozygous larvae (see Figure 2 for the structure of this double transposon), and the parent, single transposon line *B15* was examined. *B15* heterozygous flies have red eyes, and the double transposon derivative, *B14* heterozygous flies have yellow eyes, suggesting that the *white* promoter is repressed in *B14* flies. β-Galactosidase expression was greatly reduced in all imaginal discs from heterozygous *B14* larvae compared to *B15* heterozygous larvae (eye discs and haltere discs are shown in Figure 6, a–d). This result suggests that expression from the *en* promoter can be repressed by the presense of two *en* fragments.

β-Galactosidase levels in larval tissues from transposon insertions at *esg* were examined to test the generality of this result. The *esg* gene is expressed in all imaginal tissues (HAYASHI *et al.* 1993), and β-galactosidase is expressed in all imaginal tissues in larvae from lines *G6*, *G6-28* and *H4* (*G66* not examined). In this case, the expression of β-galactosidase in larvae was assayed in two types of experiments: (1) staining of larval tissues and (2) quantitation of β-galactosidase levels in homogenized larvae. In both assays, β-galactosidase levels were higher in homozygous than heterozygous larvae; and the double transposon derivative, *G6-28* had a higher β-galactosidase expression level than the single transposon parent, *G6*. The quantitative data are shown in Table 7. Homozygous *G6* and *G6-28* larvae have about two times the activity of the heterozygous larvae, as expected. Unexpectedly the level of β-galactosidase expression in *G6-28* larvae is only slightly higher (about 20%) than that of *G6* larvae, even though it has two times the number of *lacZ* genes (see Figure 2 for the structure of *G6-28*). In eye imaginal discs from *G6*, *lacZ* is expressed in a ringlike pattern similar to the expression pattern of *white* in the eye of *G6*, *3-11D*, *6-3A* and *6-2A* heterozygotes (Figure 6e). This patterned *lacZ* expression is seen both in eye discs from larvae which form adults with completely white eyes (*G6-28* heterozygotes, *G6* and *G6-28* homozygotes; a *G6* homozygous eye disc is shown in Figure 6e) as well as in *H46* eye discs where the adults have unpatterned eyes. Thus in these eye discs, expression of *lacZ* from the *en* promoter is apparently regulated independently of the *white* promoter. Independent expression patterns of *white* and *lacZ* was also noted in embryos from some *P[en]* lines (data not shown).

DISCUSSION

Pairing-sensitive sites of *engrailed* and *escargot*: Three non-overlapping fragments of *en* DNA and a fragment of *esg* DNA were identified which mediate pairing-sensitive suppression of *white* gene expression in the *P* element vector CaSpeR. The three *en* PS sites are located within 2 kb of the *en* promoter. The *esg* PS site is located within a fragment which extends 1 kb upstream of the *esg* start codon. Although the initiation site for *esg* transcription is unknown, it must be between the *P[en]* insertion site at base pair 682 and the ATG at base pair 1000 (see Figure 4), since *esg* is transcribed normally in homozygous *G6* embryos (data not shown).

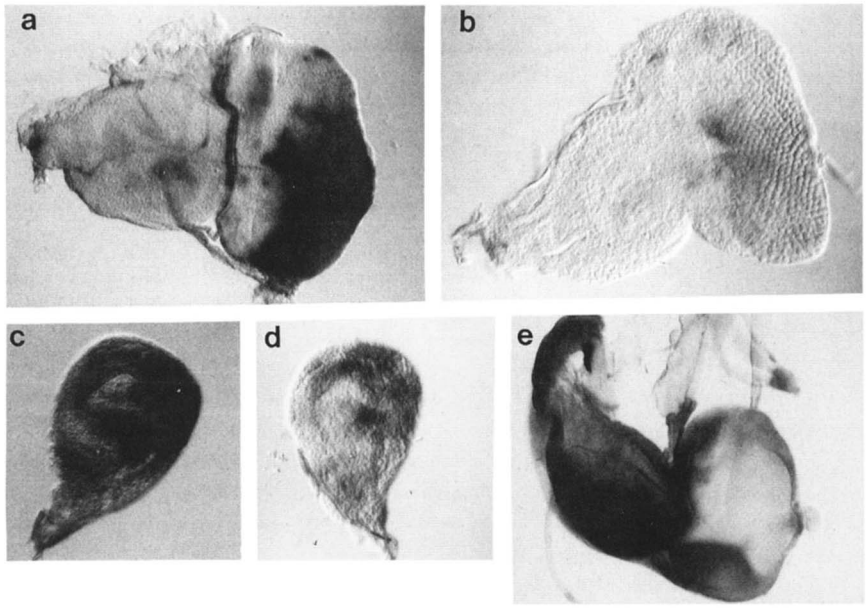

FIGURE 6.—β-Galactosidase expression in imaginal discs. Eye-imaginal discs from (a) *B15* and (b) *B14* heterozygous larvae. Haltere discs from (c) *B15* and (d) *B14* heterozygous larvae. (e) Eye-imaginal disc from *G6* homozygous larva.

TABLE 7

β-Galactosidase activity in larvae

Genotype	β-Galactosidase activity[a]
G6/+	1
G6/G6	2.1
G6–28/+	1.2
G6–28/G6–28	2.8

[a] Ratio of β-galactosidase activity in larvae of stated genotype to activity in *G6/+* larvae.

Pairing-sensitive suppression of *white* expression could be due to the interaction of the same protein with all PS sites. Thus, sequence comparison between the four PS-containing fragments might have identified a DNA sequence shared by all four fragments. However, no sequence similarities at the level of 6 of 8 bp were found. There are many possible reasons why no candidate binding site was identified by sequence comparison: (1) different PS sites may bind different proteins, (2) sequence analyses were not sophisticated enough to detect the sequence motif shared by the four fragments, (3) the aspect(s) of the sites which allows recognition has 2° or 3° structural element or 4) this phenomenon is not protein-mediated. Further subdivision of the fragments which include PS sites may help resolve this issue.

Selective insertion of *P[en]*: Wild-type P elements insert in the genome nonrandomly, but with a large number of targets (KIDWELL 1986; SPRADLING 1986; ENGELS 1989). We have previously reported that *P* transposons containing *en* sequences insert in the genome selectively (KASSIS *et al.* 1992). In that study, a *P[en]* transposon (with *en* sequences extending from −2.4 kb to +188 bp fused to the reporter gene *lacZ*) was used in an enhancer-detection screen which generated 143 lines. Only lines that expressed *lacZ* in an interesting pattern in embryos were saved and analyzed further. It was found that *P[en]* transposons insert at a high frequency (13%) in genes expressed in striped patterns during embryogenesis. The most frequent insertion site was 48A, the region of the *en* gene. In separate studies in my laboratory, we have generated a total of 110 transgenic lines using 15 different constructs containing *en* sequences extending from −2.4 kb to +188 bp and subsets thereof (constructs 1–12 in this report, A–H in KASSIS *et al.* 1991). We have maintained all lines, regardless of the embryonic expression pattern, and identified the *P[en]* insertion site. Four of these 110 lines (about 4%) are independent *P[en]* insertions at the gene *esg*. This is an extraordinarily high rate. By comparison, the 90D subdivision was the hottest spot for *P* element insertion in two large enhancer-detection screens: 14/4600 lines (0.3%) had insertions at 90D (BIER *et al.* 1989; BELLEN *et al.* 1989, 1992). *P[en]* transposons inserted at *escargot* 13 times more frequently than this; out of 4600 *P[en]* lines, one would predict 184 lines with insertions at *esg*.

Each of the four independent *P[en]* insertions in *esg* contains a different *P[en]* construct (see Figure 4). The transposon present in line *6-6B* (as well as in derivatives *6-3A* and *6-2A*) contains a 240-bp *en* fragment that is a subset of the 784-bp fragment present in Construct 3, the transposon in line *3-11D*. The transposon present in line *H46* contains a different fragment of *en* DNA, extending from −400 to +188 bp. This suggests that at least two sites within upstream *en* sequences may target transposons to *esg*. The transposon present in line *G6* contains both these sites.

P[en] transposons also insert frequently at 48A, the region of *en*. Out of the 110 independent *P[en]* insertions, three are in 48A. These three insertion events also involved three different *P[en]* constructions: line *F4* has *en* sequences from −2.4 kb to +188 bp; line *4-12E* has *en* sequences from −1944 to −1166 bp; and line *6-16C*

contains *en* sequences from −1184 to −944. In contrast to the insertions at *esg* that occurred within 260 bp of each other, the insertions at *en* are much more widely distributed, over a 200-kb region (data not shown). HAMA *et al.* (1990) also reported selective insertion of *P[en]* elements. Their *P[en]* transposons contained additional *en* sequences extending up to 7 kb upstream of the *en* promoter, and inserted in the region of 47F-48A at the incredible frequency of 7 of 20 events. Thus, larger amounts of *en* sequences seem to increase the frequency of *P[en]* insertion at *en*. This may indicate the presence of additional "selective insertion" sites in *en* DNA extending from −2.4 to −7 kb upstream of the *en* transcription start site.

PS sites and sites that cause selective insertion of *P[en]* are tightly linked, but apparently separable: In this study three PS sites were identified within a 1.6-kb region of *en* DNA. Two of these sites seem to be strong: 77% of the lines with insertions of construct 4 and 86% of insertions with construct 8 exhibit pairing-sensitive suppression of the *white* gene. In contrast, only one of six single insertions of construct 6 exhibit pairing-sensitive suppression, and only after the deletion of *P* element sequences (line *6-2A*). Thus, construct 6 seems to contain a weak PS site.

Which of the *en* fragments target insertion of *P* elements? This question is more difficult to answer since the number of lines examined for each construct is not large and the exact frequency of selective insertion of *P[en]* is unknown. One way to measure the frequency of *P[en]* selective insertion is to monitor *lacZ* expression patterns in embryos: about 13% of *P[en]* insertions expressed *lacZ* in striped patterns in embryos *versus* 3.6% from another enhancer-detector study, *P[lacW]* (KASSIS *et al.* 1992; BIER *et al.* 1989); therefore, the frequency of selective insertion may only be about 10% of the total insertion events. Thus, for any single construction, enough lines have not been obtained to answer the question of whether selective insertion is occurring. However, it is intriguing that out of five independent insertions of construct 6, one insertion occurred in *esg* and one occurred near *en* (48A).

Construct H, which contains *en* sequences extending from −400 bp to +188 bp does not contain a PS site (0/16 *P[en]* lines with this fragment (constructs 1, H and I) exhibited pairing-sensitive suppression of *white*). Yet, a very small enhancer detection study seemed to indicate that these *en* sequences did mediate selective insertion of *P[en]* transposons: 5 of 17 lines had *lacZ* expression in stripes in embryos (KASSIS *et al.* 1992). In addition, one construct H insertion was in the *esg* gene (line *H46*). This line does not behave as if it has a PS site. I reasoned that, like construct 6, a deletion of *P* element sequences from line H might cause a weak PS site to become activated. I treated line *H46* with the *P* element transposase and screened for flies with patterned eye colors. No such flies were obtained. Thus, I have not obtained evidence that construct H contains even a weak PS site by this assay.

Finally, *esg* DNA, which is a target for *P[en]* insertion, contains a PS site. This seems like a remarkable coincidence since there are few reports of this phenomenon in the literature. Recently, however, DNA fragments with PS sites have been found in regulatory DNA from *polyhomeotic* (FAUVARQUE and DURA 1993), *Sex combs reduced* (J. GINDHART and T. C. KAUFMAN, personal communication), *Proboscipedia* (A. KAPOUN and T. KAUFMAN, personal communication), *decapentaplegic* (R. PADGETT, J. SEKELSKY and W. GELBART, personal communication) and the bithorax complex (M. MULLER, H. GYURKOVICS and P. SCHEDL, personal communication). In addition, a DNA fragment from the chicken β-globin gene, which is capable of insulating *white* expression from flanking genomic enhancers in transgenic flies, was also found to suppress *white* expression in homozygous transformants (CHUNG *et al.* 1993). I propose that DNA fragments which serve as nucleation sites for large protein complexes can behave as PS sites in CaSpeR-based vectors (see below).

What is the relationship between patterned eye color and PS sites? Many of the lines derived from *P[en]* constructs have patterned eye colors. Patterned eye colors seem to be correlated with the presence of PS sites since most *P[en]* constructions that have a PS site give rise to lines with patterned eyes. However, this correlation is not absolute: no lines with patterned eye colors were derived from construct 7 and 2/21 lines that contain *en* sequences from −400 to +188 have patterned eye colors (from constructs 1, H and I). There are two problems with assessing the correlation between patterned eye colors and PS sites: first, the frequency of obtaining a patterned eye color, even with the *en* fragment which contains all three PS sites, is only 36%; and second, the frequency of appearance of patterned eye colors in CaSpeR transformants is unknown. This frequency has been estimated for the *P[lacW]* transposon, which contains the same mini-*white* gene as CaSpeR (BIER *et al.* 1989). JAMES KENNISON (personal communication) has recovered four lines with patterned eye colors from an estimated 416 insertions. Thus there are rare insertion sites in the Drosophila genome that result in patterned eye colors even in the absence of transposon-encoded PS sites.

The correlation between a PS site and a patterned eye color can best be made by examining the insertions at *esg*. Even in this case, the results are somewhat complicated. Insertion of constructs G, 3 and 6 (which all contain at least one PS site) have patterned eye colors; whereas insertion of construct H (which does not contain a PS site) leads to an evenly colored eye. This result is complicated by line *G66*, which contains transposon G with three PS sites, but does not have patterned eyes.

In line *G66*, 3.5 kb of *esg* DNA have been deleted (including about 300 bp of those present in *P[esg]*). These sequences are apparently necessary to produce a patterned eye color.

The insertion in line *G66* cannot interact to suppress eye color when combined with any other transposon (see Table 3). This suggests that the deletion of *esg* sequences effectively inactivates the *P[en]*-PS sites present in line *G66*. Note that this lack of interaction is not due to the orientation of the transposon in *esg* DNA: the transposons present in lines *6-3A* and *6-2A* are in the opposite orientation to those present in *3-11D* and *G6*, but still interact with these transposons to reduce eye color. Thus it seems that the presence of the deleted *esg* sequences are crucial for both a patterned eye color and for suppression of the *white* gene. My hypothesis is that a PS site has been deleted from the *esg* DNA, and that an interaction between the *esg*- and the *P[en]*-encoded PS sites is crucial both for patterning and suppression of *white*. Alternatively, deletion of the *esg* DNA may have introduced flanking DNA which inhibits the interactions necessary to produce a patterned eye color and pairing-sensitive suppression of *white*.

Similarities between *P[en]* and *P* elements that contain the *white* gene with *zeste* binding sites: The endogenous *white* gene responds to the *zeste* mutation, z^1, in much the same way as *P[en]* behaves in a wild-type background; z^1 suppresses the expression of the *white* gene in the eye of the fly in a proximity-dependent manner. Interactions occur between two copies of *white* on paired homologous chromosomes and on two copies of *white* in close proximity on the same chromosome (GUBB *et al.* 1986; HAZELRIGG 1987). Zeste protein binds DNA and stimulates *white* transcription *in vitro* and *in vivo* (BENSON and PIRROTTA 1988; MANSUKHANI *et al.* 1988; BIGGIN *et al.* 1988; LANEY and BIGGIN 1992). The zeste protein self-aggregates into large complexes; this aggregation is required for transcriptional activation (BICKEL and PIRROTTA 1990; PIRROTTA 1991). The z^1 mutation, which results from a single amino acid change, causes the zeste protein to form larger multimeric complexes (CHEN and PIRROTTA 1993). These larger complexes are thought to interfere with *white* transcription. The DNA fragments necessary for the z^1-*white* interaction have been identified by *P* element transformation experiments. Transposons with 11.7 kb of the *white* gene (*P[w]*) retain their ability to respond to z^1 (HAZELRIGG *et al.* 1984).

The similarities in the pairing-sensitive suppression of *white* seen with *P[en]* to the z^1 interaction with *P[w]* is striking. First, both interactions are very sensitive to position effects. *P[w]* responds to *zeste* in some chromosomal positions, but not in others, and the magnitude of the response varies (HAZELRIGG *et al.* 1984). Likewise, repression of *white* expression from *P[en]* in homozygotes is complete in some locations, while at others the eye color of homozygotes is indistinguishable from the eye color of heterozygotes. At still other locations, no suppression is seen. Another similarity of *P[en]* and *P[w]* is the production of patterned eye colors in some genomic locations. Strikingly, *P[w]* lines with patterned eyes showed repression by z^1 even in heterozygotes, suggesting that another zeste binding site may be nearby in the genome (HAZELRIGG *et al.* 1984; HAZELRIGG and PETERSEN 1992). Similarly, *P[en]* insertions at *esg* have patterned eye colors, and *esg* has a PS site. Finally, z^1 mutations often interact with *P[w]* to give variegated eye colors (HAZELRIGG *et al.* 1984). Pairing-sensitive suppression of *white* also leads to varying degrees of variegation, and some *P[en]* heterozygotes have variegated eyes (see the eyes of A2/+ and A2/E7 flies in Figure 3B of KASSIS *et al.* 1991).

The similarities in the behavior of *P[en]* and *P[w]* elements leads me to propose that PS sites bind a large protein complex that represses *white* transcription in the same way that mutant zeste proteins repress *white* transcription. The four PS sites identified may bind the same complex or they may be nucleation sites for different protein complexes.

The position of insertion in the genome determines which Polycomb and trithorax group genes can influence *white* expression from *P[en]*: We have previously shown that *zeste* mutations do not affect the pairing-sensitive suppression of *white* from *P[en]* (KASSIS *et al.* 1991). Here, Polycomb and trithorax group mutations were tested for their effect on *white* expression from *P[en]* for two reasons: (1) some Polycomb group mutations are known to suppress or enhance the effect of z^1 on *white* expression and thus are thought to influence transvection (WU and GOLDBERG 1989; PIRROTTA 1990; WU 1993; RASTELLI *et al.* 1993) and (2) it has been proposed that proteins encoded by the Polycomb group genes form large complexes that repress transcription of many genes, including homeotic genes and *en* (PARO 1990; MOAZED and O'FARRELL 1992). Some trithorax group gene products, which have been genetically identified as activators of homeotic gene activity, may also encode proteins that form complexes (KENNISON 1993). Some Polycomb and trithorax group mutations affected *white* expression from *P[en]*. However, no mutation affected *white* expression from all insertion sites, and different mutations affected expression from different insertion sites. The results suggest that the effects of these mutations are not mediated through the *en* fragment directly, but rather, are a consequence of the action of Polycomb and trithorax group genes on sequences flanking the insertion sites of the *P[en]* transposons. In support of this view, consider the following: the *en* fragment extending from −2.4 to +188 bp does not contain tissue-specific enhancer activity: it simply acts as an enhancer trap (KASSIS 1990; KASSIS *et al.* 1992). In some *P[en]* lines that also contain the *lacZ* as as reporter, *lacZ*

is expressed in the eye disc in a pattern that resembles the pattern of color in the eye. This patterned expression is dependent on the position of insertion in the genome. Thus, it seems likely that *lacZ* and *white* expression may be stimulated (or repressed) by flanking genomic regulatory sequences, and that mutations that influence the expression of flanking genes may influence the expression of *white* from *P[en]*.

Since it has been proposed that the Polycomb group genes all act together in a complex (PARO 1990), it is curious that *Pcl* affected the expression of the *white* gene in *B14* flies whereas other members of this group had no effect. This could mean that different members of the group act at different locations. In support of this, three Polycomb group proteins: *Psc*, *ph* and *Pc* have been found to bind to specific polytene chromosome bands (ZINK and PARO 1989; DECAMILLIS *et al.* 1992; MARTIN and ADLER 1993) while the binding sites for *ph* and *Pc* are reported to be identical (DECAMILLIS *et al.* 1992), those for *Psc* overlap with, but are not identical to those of the other proteins, suggesting that these proteins may act independently at some sites (RASTELLI *et al.* 1993).

How do the PS sites inactivate expression? It was recently suggested that a fragment of *polyhomeotic* (*ph*) regulatory DNA causes a local heterochromatization, which represses gene transcription (FAUVARQUE and DURA 1993). This 2.6-kb fragment, when cloned into a CaSpeR-based vector (*P[ph]*), imparts properties strikingly similar to those of *P[en]* transposons containing the *en* fragment extending from −2.4 to +188 bp: transformant lines derived from *P[ph]* have a high frequency of patterned and variegated eye colors, *white* expression is repressed in *P[ph]* homozygotes and *P[ph]* transposons insert in the genome selectively. Even some of the details of the *en*- and *ph*-mediated phenomena are quite similar: the frequency of lines with patterned eye colors (38% for the *ph* fragment, 36% for the *en* fragment), and one of the targets for insertion, 35C (*esg*), are apparently the same (FAUVARQUE and DURA do not indicate whether their insertions are in *esg*). FAUVARQUE and DURA have suggested that the *ph* DNA causes a local heterochromatization, leading to the inactivation of both *lacZ* and *white* expression. Although this may be the case, this local heterochromatization is highly dependent on interactions with flanking DNA as only 36% of their lines have a patterned eye color. Also, this fragment of *ph* DNA is apparently unable to inactivate a heat shock promoter, suggesting that local heterochromatin may be too strong a term.

The data from my laboratory suggest that, for both the *white* and the *en* promoter (driving *lacZ*), repression is highly dependent on the position of insertion in the genome. I favor a model that is similar to that of *zeste*-mediated repression of *white*. I propose that, at the *en* locus, the PS sites normally act to bring together distant pieces of regulatory DNA to activate (or repress) *en* transcription. In *P[en]* transposons these PS sites act with flanking genomic PS sites to either loop out the *white* gene, or the *lacZ* gene (or both) and that the promoters are inactivated in homozygotes due to interference by a protein complex which excludes communication between positive regulators and the promoter.

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