Differential Gene Silencing by *trans*-heterochromatin in *Drosophila melanogaster*

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ABSTRACT

The *brown*^{Dominant} (*bw*^{D}) allele contains a large insertion of heterochromatin leading to the *trans*-inactivation of the wild-type allele in *bw*^{D}/*bw*$^{+}$ heterozygous flies. This silencing is correlated with the localization of *bw*$^{+}$ to a region of the interphase nucleus containing centric heterochromatin. We have used a series of transgene constructs inserted in the vicinity of the *bw* locus to demarcate both the extent of *bw*^{D} influence along the chromosome and the relative sensitivities of various genes. Examples of regulatory regions that are highly sensitive, moderately sensitive, and insensitive were found. Additionally, by using the same transgene at increasing distances from the *bw*^{D} insertion site *in trans* we were able to determine the range of influence of the heterochromatic neighborhood in terms of chromosomal distance. When the transgene was farther away from *bw*, there was, indeed, a tendency for it to be less *trans*-inactivated. However, insertion site also influenced silencing: a gene 86 kb away was *trans*-inactivated, while the same transgene 45 kb away was not. Thus location, distance, and gene-specific differences all influence susceptibility to *trans*-silencing near a heterochromatic neighborhood. These results have important implications for the ability of nuclear positioning to influence the expression of large blocks of a chromosome.

CHROMOSOMAL rearrangements that place a gene next to the highly condensed heterochromatin can result in spotty, epigenetic silencing of gene transcription called position-effect variegation (PEV). Usually, PEV alleles of Drosophila behave as simple loss-of-function mutations and, unless haplo-insufficient, are recessive. However, some genes are susceptible to heterochromatic rearrangements *in trans*, a phenomenon called *trans*-inactivation. An example of a gene that can be *trans*-inactivated is the *brown* (*bw*) gene that encodes a membrane transport protein necessary for the deposition of red eye pigment. All PEV alleles of *bw* (generally referred to as *bw*^{V} alleles) are dominant to an unrearranged *bw*$^{+}$ chromosome. An essential requirement of *bw*^{V} variegation is the somatic pairing of homologs. The dependence of variegation on somatic pairing is demonstrated by *bw*$^{+}$ transgenes that are inserted at ectopic positions. These transgenes do not pair with the endogenous locus and consequently are dominant to *bw*^{V} rearrangements (Dreesen *et al.* 1991).

The *brown*^{Dominant} (*bw*^{D}) allele is caused by the insertion of ~1.6 Mb of the heterochromatic repeat AAGAG into exon 7 of the *bw* coding sequence, which is located near the distal end of the right arm of the second chromosome (2R) at polytene band 59E (Platero *et al.* 1998). The insertion results in an otherwise unrearranged chromosome with a large block of heterochromatin ~19 Mb away from the bulk of the centric heterochromatin. The *bw*^{D} allele causes *trans*-inactivation of *bw*$^{+}$, with *bw*^{D}/*bw*$^{+}$ heterozygotes containing only ~2% of the wild-type red eye pigment. The *trans*-inactivation phenotype consists of a few scattered wild-type ommatidia in an otherwise null mutant background. *bw*^{D}/*bw*$^{+}$ heterozygotes show an exclusively "salt and pepper" phenotype, whereas some other PEV alleles show a more sectored phenotype with contiguous cells tending to have the same expression state. Nevertheless, the salt and pepper phenotype of *bw*^{D}/*bw*$^{+}$ can be phenotypically suppressed by classic *Su(var)* mutations defined by their effect on classic *cis*-PEV (Weiler and Wakimoto 1995; Sass and Henikoff 1998).

The classic model for PEV involves the linear spreading of a heterochromatic chromatin structure from the euchromatin-heterochromatin boundary into the variegating gene (Spofford 1976). Such a strict linear mechanism is obviously not satisfactory for *trans*-inactivation. However, there does seem to be a relationship between the centric heterochromatin and *bw*^{D} *trans*-inactivation. When chromosomal rearrangements bring the *bw*^{D} locus closer to, but not contiguous with, heterochromatin, *bw*^{D} *trans*-inactivation is enhanced. The inverse is true for those rearrangements that moved *bw*^{D} farther from heterochromatin (Talbert *et al.* 1994; Henikoff *et al.* 1995). This change in the strength of *bw*^{D} PEV with distance could not be due to increased or decreased linear spreading of heterochromatin from the centric regions, since megabases of euchromatin remained unaltered and active between *bw*^{D} and centric heterochromatin in both the enhanced and suppressed lines. This distance-dependent enhancement and suppression of *bw*^{D} is very similar to a heterochromatin distance effect

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described for genes normally resident in heterochromatin (Wakimoto and Hearn 1990; Eberl *et al.* 1993). These observations gave rise to a compartmentalization model where proteins necessary for the correct expression of different types of genes were postulated to be concentrated in specific regions of the interphase nucleus. It was hypothesized that heterochromatic genes required a nuclear neighborhood of heterochromatic proteins, while euchromatic genes required a euchromatic neighborhood. Direct evidence for bw^D heterochromatic compartmentalization was found when fluorescence *in situ* hybridization (FISH) to interphase diploid nuclei showed that *trans*-inactivation is correlated with the close association of the *bw* locus and the centric heterochromatin of 2R (2Rh). The correlation between the nuclear position and the silencing of bw^+ led to the proposal that the dominant effect of bw^D is due to the dragging of the somatically paired homolog into the heterochromatic compartment of the nucleus where it is silenced (Csink and Henikoff 1996). Dominant PEV may include a step that is fundamentally different from classic *cis*-PEV, since heterochromatin cannot spread linearly along the chromosome from the heterochromatic nucleation site on the homolog to the inactivated gene. Alternatively, *cis*-PEV may also result from mislocalization to the heterochromatic neighborhood.

Variegating alleles of *bw* are consistently dominant. Therefore, if the compartmentalization model is correct, the regulation of the bw^+ gene must be particularly sensitive to the heterochromatic compartment compared to other genes commonly used to study PEV. However, there is increasing evidence that other genes are sensitive to heterochromatin *in trans*. *white* transgenes with modified regulatory sequences have been shown to be sensitive to heterochromatin *in trans* in a number of instances, although *white* in its endogenous location is not. Additionally, an unidentified vital gene *in trans* to a block of heterochromatin was inactivated (Martin-Morris *et al.* 1997). Perhaps a qualitative or quantitative aspect of *bw* transcription renders it sensitive to *trans*-inactivation. For instance, *bw* transcript abundance does not increase until late in pupal development, while *white* transcript abundance increases earlier (Bhadra *et al.* 1997). The difference in susceptibility to *trans*-inactivation for *bw* and *w* may be explained by transcriptional activation of *w* but not *bw* before the heterochromatic compartment has been fully established (Csink and Henikoff 1998). Alternatively, the fact that the removal of enhancer sequences from the *w* locus can allow *trans*-inactivation supports the idea that relatively weak transcription can render a gene more sensitive to the heterochromatic compartment (Martin-Morris *et al.* 1997).

In this article we address questions concerning differential effects of heterochromatin *in trans* on various genes using a series of transgenes inserted in the *bw* region. We have used this series to demarcate both the extent of bw^D influence along the chromosome and the relative sensitivities of the various transgenes themselves. Examples of regulatory regions that were highly sensitive, moderately sensitive, and insensitive were found. Additionally, by using the same transgene at increasing distances from the bw^D insertion site *in trans* we were able to determine the range of influence of the heterochromatic neighborhood in terms of chromosomal distance. When the transgene was farther away from *bw*, there was, indeed, a tendency for it to be less *trans*-inactivated. Interestingly, insertion site also influenced silencing: a transgene 86 kb away from the *bw* locus was *trans*-inactivated, while it was not at 45 kb. Finally, different reporter genes in identical positions responded differently to heterochromatin *in trans*. Thus location, distance, and gene-specific differences all influence susceptibility to *trans*-silencing near a heterochromatic neighborhood. These results have important implications for our understanding of how nuclear positioning can influence the expression of large blocks of a chromosome.

MATERIALS AND METHODS

Fly stocks: *P*-element lines shown in Table 1 containing inserts in lethal loci were obtained from either the Bloomington Stock Center or the Berkeley Drosophila Genome Project (BDGP). Some of these lines are no longer maintained by either source, but can now be obtained from us. Lines s4830, k06908, 08856, 01862, k05606, k11531, 03041, and k15608 were previously described as verified, in that they are lethal over a deficiency for the region of the *P*-element insert or fail to complement another lethal line mapped to the same cytological location (Spradling *et al.* 1999 and BDGP). The complementation analysis for the various alleles of the *Dcp-1* and *apt* genes is summarized on FlyBase (http://flybase.bio.indiana.edu/).

Sequences for all *P*-element insertion sites, except two, are available through BDGP or can be found in the specific gene summaries on FlyBase. The insertion site and orientation in lines 08859 and k05606 was determined using inverse PCR and flanking PCR, respectively.

The lines containing *P{hsp-w-hsp26-pt-T}* on the X chromosome were kindly provided by L. Wallrath. All other mutations are described on FlyBase (http://flybase.bio.indiana.edu/).

Crosses to assess *trans*-inactivation: *mini-white:* The wild-type Drosophila eye contains both red and brown pigments. White protein is necessary for the transport of both brown and red pigments, but brown is necessary only for the transport of red pigment. To determine if bw^D heterochromatin *in trans* could inactivate a *mini-white* gene inserted near the *bw* locus, we must be able to analyze *white* expression separately from *brown trans*-inactivation. To do this, females of the genotype w^{67c23}*;P{lacW}/CyO* were crossed to bw^D*;P{cos* bw^+*}* males. This cross eliminates transcripts from the endogenous *white* gene so we can detect expression of the *white* transgene on chromosome 2. If we then eliminate endogenous *bw* activity (by bw^D heterochromatin *in trans*), we would then modify eye color, but we would not know if this modification was due to inactivation of *bw* or inactivation of the *white* transgene. Therefore, the presence of the *bw* transgene on the third chromosome (which is unaffected by bw^D; Dreesen *et al.* 1991) ensures that eye color is not influenced by a lack of the endogenous *bw* gene product.

TABLE 1

***P*-element insertion lines tested for *trans*-inactivation by bw^D**

Insert line	Element	Direction	Gene	GeneSeen position no.[a]	Distance from bw^D insert (18466689)[b]
10444	PZ	p		17863215	603474
k09913	lacW	d		18006009	460680
k07136	lacW	p		18186492	280197
s4830	lacW	p		18382215	84474
k06908	lacW	p	chrw	18461953	4736
08859	PZ	p	Dcp-1	18489504	15313
01862	PZ	p	Dcp-1	18489565	15374
k05606	lacW	p	Dcp-1	18489846	15655
k11531	lacW	d	apt	18518850	44659
03041	PZ	p	apt	18519095	44904
k15608	lacW	d	apt	18519313	45122

[a] From the numbering given to this region on 2R in the GeneSeen data present on the BDGP website as of April 2001.

[b] The line sequenced by the Drosophila Genome Project contained the bw^1 mutation, which is not present in any of the lines in our study. bw^1 contains an insertion of the 412 retrotransposon, which is a total of 7502 bp. This 412 insertion site is distal to the bw^D insertion site. The distances for the distal *P*-element insertions have been adjusted accordingly.

The variability of expression of the *mini-white* transgene in different locations will not allow phenotypic comparison between an insertion being tested for *trans*-inactivation and an insertion at another site, so comparisons of *mini-white* expression were made between the flies carrying bw^D and flies carrying bw^+ for each insert. All flies were aged 10–13 days before photography. At least 200 bw^D/*mini-white* flies were examined for each insertion to assess the amount of phenotypic variation and to ensure that, in the event that no *trans*-inactivation was apparent, rare variegation events would not be missed.

P{hsp-w-hsp26-pt-T}: Female w^{1118};*P{hsp-w-hsp26-pt-T}/CyO* were crossed to w^{1118};bw^D. Cy^+ male progeny containing the transposon at *Dcp-1* or *chrw* were compared to those carrying the transposon at a site distant from 59E.

To examine the consequence of the loss of the Y chromosome on bw^D *trans*-inactivation of *P{hsp-w-hsp26-pt-T}*, female w^{1118};*P{hsp-w-hsp26-pt-T}/CyO* were crossed to *C(1,Y)*;bw^D and the w^{1118}/*O;P{hsp-w-hsp26-pt-T}*/bw^D male progeny were compared to w^{1118}/*Y;P{hsp-w-hsp26-pt-T}*/bw^D males. At least 500 bw^D/*P{hsp-w-hsp26-pt-T}* flies were examined for each insertion.

Larvae: w^{1118} males that contained the various transposons over a *Bc Elp* chromosome were crossed to w^{1118};bw^D or w^{1118} females. Only Bc^+ larval progeny were analyzed either by histochemical staining for β-galactosidase activity or by abundance of *w* or *pt* transcripts. All larvae analyzed were wandering late third instar.

β-Galactosidase assays: β-Galactosidase staining of larval tissues was performed as previously described (Ashburner 1989). Staining was done on wandering late third instar larvae and staining pattern was observed after 3 and 18 hr. Each genotype was examined for staining pattern in at least three larvae.

Quantitative assays using chlorophenolred-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis) were also done on adult flies as described by Ashburner (1989), except that the incubation times were 2, 4, and 8 hr. Additionally, a Bradford assay was done to determine total protein concentration and the value used to equalize the samples for total protein. Thirty flies were used for each assay, and each genotype, sex, and age was done in triplicate.

Pigment assays: Pigment assays for brown pigments were adapted from the procedure of Ephrussi and Herold (1944). Flies were sorted according to genotype and sex, aged for 4–5 days, and frozen at −70°. For each assay, six heads were collected in a 0.5-ml microfuge tube and 14 μl of acidified methanol (1 ml methanol, 3 μl 12 M HCL) was added. Pigment was extracted by rocking tubes for 48 hr at room temperature in the dark, after which 1 μl of 0.08% H_2O_2 was added to each sample and incubated for an additional 1 hr. The optical density of each sample was read in a capillary cuvette in a spectrophotometer at 470 nm and the value of a methanol blank was subtracted to give the values shown in Figure 4. Six samples from each genotype were measured.

***P*-element replacement:** Crosses used to obtain *P*-element replacements of *P{hsp-w-hsp26-pt-T}* in the *chrw* and *Dcp-1* sites are shown in Figure 1 and are similar to those described by Sepp and Auld (1999). Briefly, males carrying the donor element on the X chromosome, the target element on the second chromosome at 59E, and a transposase source in a w^- background were generated and mated to w^- females. Transpositions to the autosomes were detected by bright red eyes in male progeny. These males with putative replacements were mated to w^- females and the second chromosome was then balanced. Potential replacements at *Dcp-1* were evaluated using X-gal staining for loss of *lacZ* activity in the original target element, FISH, and Southern blotting. Potential replacements at *chrw* were assessed with recombination crosses to phenotypically expose remaining target elements. Additionally, FISH and PCR using primers annealing to sequences flanking the insert and unique to *P{hsp-w-hsp26-pt-T}* were also used to detect replacements at *chrw*. The two replacement lines chosen for further study (*P{hsp-w-hsp26-pt-T}Dcp-1*^{mgb48} and *P{hsp-w-hsp26-pt-T}chrw*^{nb28}, referred to as *Dcp-1*^{mgb48} and *chrw*^{nb28}) were analyzed using a Southern blot to assure that there were no gross rearrangements of the new *P{hsp-w-hsp26-pt-T}* element at the target site or the immediate flanking sequences.

For the replacement in *Dcp-1*, the allele *P{PZ}Dcp-1*^{8859} was used as the target and *P{hsp-w-hsp26-pt-T}39C-X* was the donor (Wallrath and Elgin 1995; Spradling *et al.* 1999). Of 3557 males screened, 114 (3.2%) contained a transposition to an autosome. Of these, 53 (1.15%) contained a w^+ gene that

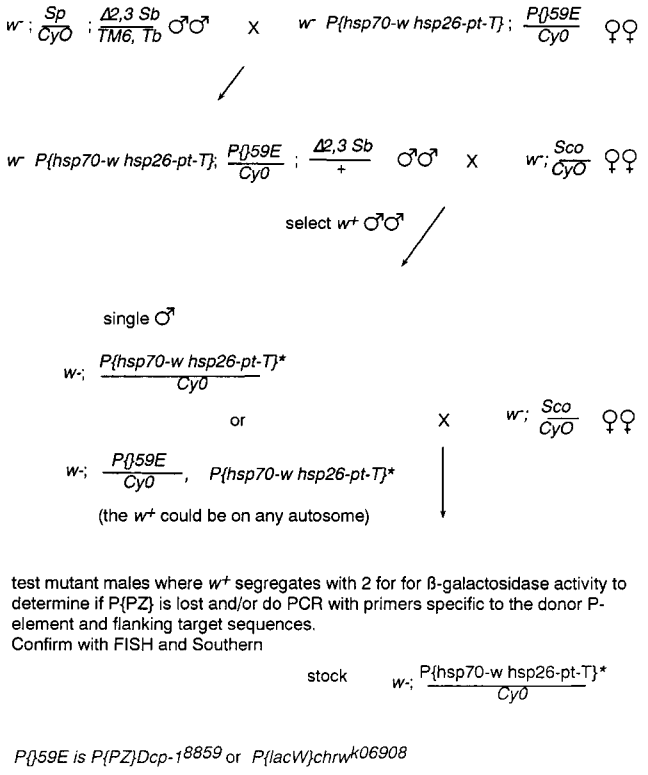

FIGURE 1.—Replacement of *P{PZ}Dcp-1* or *P{lacW} chrw* with *P{hsp-w-hsp26-pt-T}* from the X chromosome. *Sb*, *Tb*, *Sp*, and *Cy* are dominant phenotypic markers that are homozygous lethal. CyO and TM6 are balancer chromosomes. Δ2,3 is the source of the transposase to mobilize the *P*-element construct. *, a *P*-element construct mobilized to a new position.

segregated with the second chromosome and 7 of these were replacements into the *Dcp-1* locus. One of these replacements was only partially intact and another line contained a second insertion on chromosome 2 in addition to the one at *Dcp-1*. For the replacement in *chrw*, the *P{lacW} chrw*^{k06907} was the target and *P{hsp-w-hsp26-pt-T} 118E-X* was the donor. Of 4510 males screened, 133 (2.9%) contained a transposition to an autosome. Of these, 41 (0.91%) segregated with the second chromosome and 3 of these were replacements into the *chrw* locus (2 complete and 1 imperfect).

Recombination of *bw*^{D} onto *P*-element containing chromosomes: To evaluate effects of *bw*^{D} on two *P* elements *in cis*, recombinant chromosomes were created by mating *w*$^-$*;P{lacW} k11531*^{k11531}*/bw*^{D}*Pin*^{2} or *w*$^-$*;P{hsp-w-hsp26-pt-T}Dcp-1*^{mgh48}*/bw*^{D}*Pin*^{2} virgin females to *w*^{1118}*;Sco/CyO* males. *Pin*$^+$ progeny that showed some eye pigment were examined for a *bw*$^-$ phenotype. In the *P{lacW}k11531*^{k11531} screen, 6693 *Pin*$^+$ flies were screened. Four potentially recombinant females with pale peach-pink eye color were found and three were used to make balanced stocks. Among 9094 *Pin*$^+$ flies screened for recombination with *P{hsp-w-hsp26-pt-T}Dcp-1*^{mgh48}, 2 were found that had red-brown eye color and 1 of these was used to make a balanced stock. All four recombinant stocks were confirmed by concurrent multicolor fluorescence *in situ* hybridization to polytene chromosomes using the satellite repeat AAGAG, a *lacZ* probe, and a *bw* genomic clone (ASHBURNER 1989; PLATERO *et al.* 1998).

Northern blots: Total cellular RNA of the various genotypes was isolated from 30 to 50 4-day-old adult female flies or 15 female wandering late third instar larvae using the QIAGEN (Chatsworth, CA) RNeasy Mini Kit. The flies and larvae were heat-shocked in a water bath for 1 hr immediately before RNA extraction. Those that were grown at 25° were heat-shocked at 37° and those that were grown at 18° were heat-shocked at 32° (heat shock at 37° resulted in death). Following electrophoresis on 1.5% agarose formaldehyde gels, the RNA was transferred to Biotrans nylon filters (ICN). Filters were prehybridized for 4 hr and ^{32}P-labeled RNA probes added to a final concentration of 400,000–1 million cpm/ml. These were hybridized for 24 hr and exposed to a phosphorimager screen for quantitation (CSINK *et al.* 1994).

white and plant transcript probes were derived from pIBI-wDNA linearized with *Stu*I (CSINK *et al.* 1994) and pGH11 (gift of L. Wallrath) linearized with *Cla*I. pGH11 contains ~1600 bp of plant cDNA cloned into the *Not*I/*Cla*I site of pBluescript KS(+), which is the cDNA driven by the *hsp-26* promoter in *P{hsp-w-hsp26-pt-T}*. *rp49* hybridization was used to correct for gel-loading variabilities (KONGSUWAN *et al.* 1985). Each probe was transcribed using T7 polymerase to incorporate [^{32}P]UTP according to the manufacturer's instructions (Promega, Madison, WI).

Fluorescence *in situ* hybridization: FISH to diploid larval central nervous system (CNS) nuclei and the analysis of internuclear distances were performed as described previously (CSINK and HENIKOFF 1996), except the measurements were performed in three dimensions. Briefly, probes for the satellites AACAC and a P1 specific for the 59E region (Berkeley Drosophila Genome Project) were labeled with dUTP-FITC and dUTP-rhodamine (Amersham, Buckinghamshire, UK), respectively. Nuclei were visualized using a Deltavision microscope (Applied Precision) with a cooled CCD camera and recorded at ×600 original magnification. Each of the three wavelengths was corrected using the Deltavision 3D deconvolution program (Applied Precision). Using Softworx software (Applied Precision) we measured the distance between the closest FITC and rhodamine signals and the area of the 4′6-diamidino-2-phenylindole-stained nuclei. The radius of each nucleus was calculated based on the area, assuming a circular shape of the nucleus. To ensure that measurements were unbiased by the experimenters' expectations, the image files were randomly coded and the codes were not broken until all measurements were completed. From each of at least three slides for each genotype and treatment, three separate fields were randomly selected. Up to 10 randomly selected nuclei were analyzed from each field, for a total of at least 90 measurements for each genotype. If additional data were gathered it was from additional larval slide preps, so that there were never >30 nuclei from a single larva in a data set. All larvae were male and wandering late third instar. Larvae were heat-shocked for 30 min at 37° immediately prior to dissection. Such treatment was confirmed as resulting in a heat-shock response by staining larvae containing an *hsp70-lacZ* transgene for increased β-galactosidase activity.

RESULTS

***Trans*-inactivation of *mini-white* in single insertion lethal lines:** The *P*-element transposon *P{lacW}* contains a *mini-white* transgene and a *lacZ* gene driven by the minimal *P*-element promoter region (Figure 2A). The gene *white* codes for a transmembrane protein involved in the transport of both the brown and red eye pigments. *mini-white* is driven by a regulatory region that lacks many of the tissue-specific enhancers from the endogenous gene and thus its expression is lower than a trans-

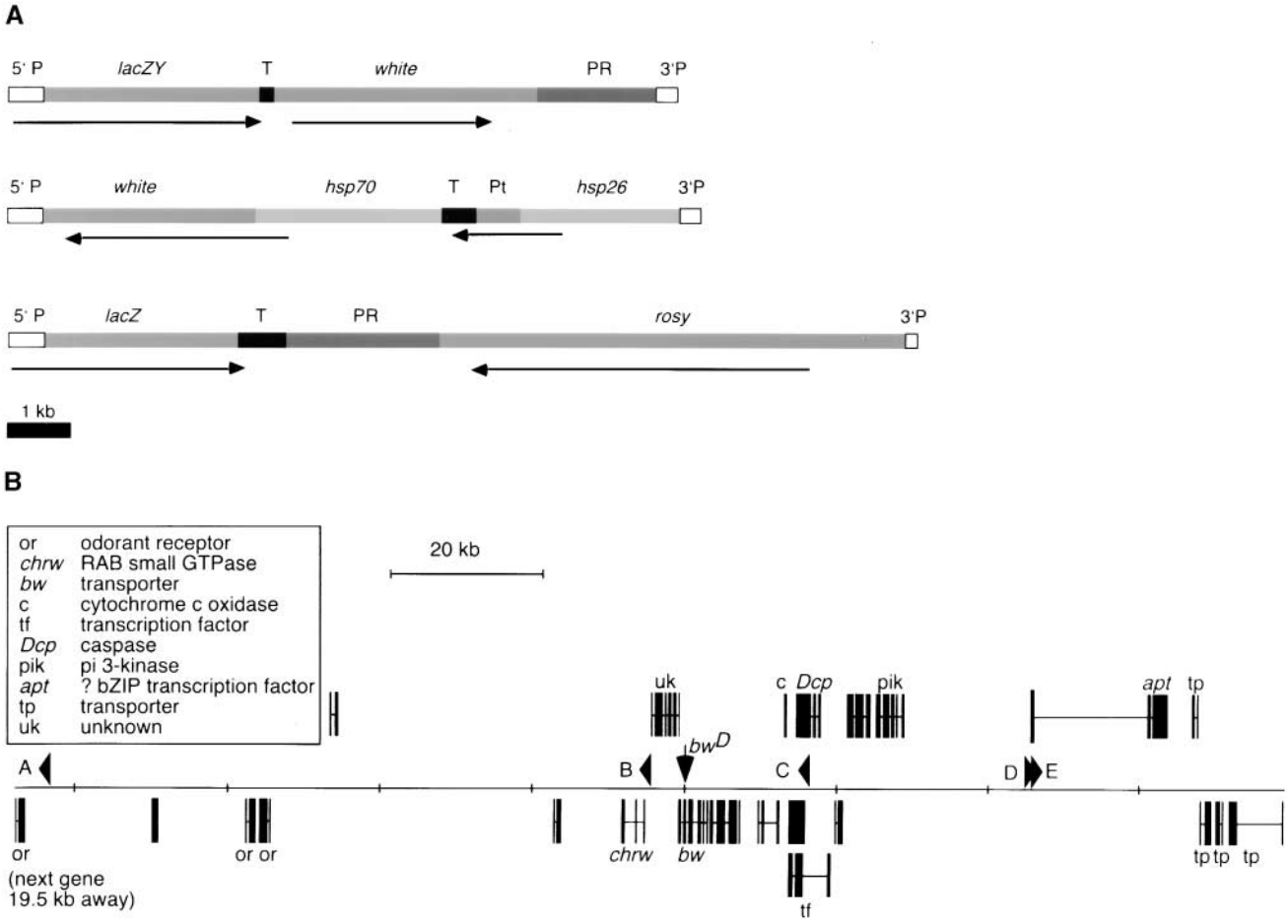

FIGURE 2.—(A) Three *P*-element transposons used in this study. From top to bottom: *P{lacW}* (BIER *et al.* 1989), *P{hsp-w-hsp26-pt-T}* (WALLRATH and ELGIN 1995), and *P{PZ}* (SPRADLING *et al.* 1999). *lacZ*, *lacZY*, *white*, *pt*, and *rosy* are reporter genes. These reporter genes are driven by minimal *white*, *hsp26*, *hsp70*, the *P*-element (in 5′ P sequence), and *rosy* promoters. Arrows indicate transcripts. T, termination sequences; PR, plasmid rescue sequences. (B) Transcription map of the region around the *brown* locus. The map is adapted from the GeneSeen output at the Berkeley Drosophila Genome Project website (http://www.fruitfly.org/annot/). Putative transcription units are shown above and below the line and descriptions of selected loci are in the box. Only putative genes that contain at least an EST hit or a region of homology with another gene are included. Putative genes with only Genefinder or Genie support were not included in the figure (seven in all). Because of the scale of the figure a number of introns and exons are drawn as contiguous. The *apt* transcript was modified to include information from published articles not annotated in GeneSeen (EULENBERG and SCHUH 1997; LIE and MACDONALD 1999). The Genome Project sequenced the bw^1 allele, which is caused by the insertion of a 412 element. This was removed in the figure. The arrow points to the site where heterochromatin is inserted on the bw^D chromosome. The arrowheads indicate the insertion sites of the *P{lacW}* *P* elements shown in Figure 3 in the same order and have been given letter designations for easier reference in the text. A, s4830; B, k06908; C, k05606; D, k11531; and E, k15608. The arrowheads point in the 5′ to 3′ direction of the *P* element.

gene containing a full regulatory region. Additionally, *mini-white* is subject to chromosomal position effects (BIER *et al.* 1989). Depending on its euchromatic site of insertion, its phenotype can range from very pale yellow (or absent) to a deep brownish red. A single copy never shows full pigment, defined by the appearance of a pseudopupil. The variation of pigment has been interpreted as a readout of either the interaction of the *mini-white* promoter with local enhancers or the sensitivity of this reporter to the overall openness of the local chromatin. Previous studies have shown that *mini-white* is sensitive to heterochromatin in both *cis* (WINES *et al.* 1996) and *trans* (MARTIN-MORRIS *et al.* 1997). *Trans*-inactivation was shown both for an inversion with a heterochromatic breakpoint and a repeat-array-induced block of heterochromatin.

We wished to extend these results to the bw^D heterochromatic insertion and to use them to determine the distance a gene could be from *bw* and still show *trans*-inactivation. In addition, we wished to see if there were local effects that would influence the susceptibility of a gene to this type of gene silencing or whether distance would be the only determining factor. A series of *P{lacW}* insertions mapping close to the *bw* locus was

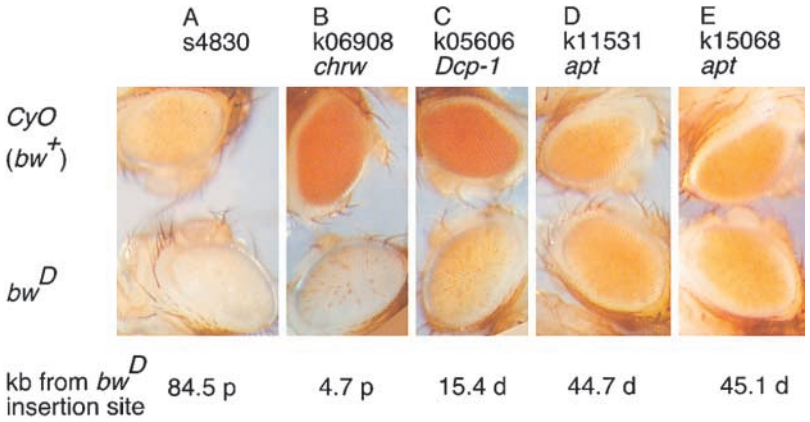

FIGURE 3.—*Trans*-inactivation of *mini-white* by bw^D. Flies (aged 10–13 days) of the genotypes yw^{67c23};*P{lacW}/CyO* (top row) and yw^{67c23};*P{lacW}/* bw^D;*P{cos bw+}/+* (bottom row). The insertion designation from the BDGP gene disruption project (http://www.fruitfly.org/p_disrupt/) is shown above each picture and the gene into which it is inserted is shown, if known. The letter designations refer to the insertion site designations in Figure 2B. Below the photos is the distance from the bw^D insertion site. p, proximal, d, distal.

tested for *trans*-inactivation by bw^D. Lines carrying these insertions are from a collection of single element homozygous lethal chromosomes created, mapped, and maintained by the Drosophila Genome Project and Bloomington Stock Center (SPRADLING *et al.* 1999). Figure 2B shows the location of five of these insertions relative to various known and putative genes within the region.

Four of the seven *P{lacW}* insertion lines tested (Table 1) showed *trans*-inactivation of the *mini-white* gene in the *P{lacW}* transposon (Figure 3). The distance of these transposons from the site of the bw^D insertion is given in Table 1 and the position of five of these inserts relative to genes and putative genes identified by the Drosophila Genome Project is shown in Figure 2B. The eyes of the four lines that showed *mini-white trans*-inactivation as well as one that did not are shown in Figure 3. Two other *P{lacW}* lines, k09913 and k07136, 460 and 280 kb proximal to the bw^D insertion site, respectively, were not *trans*-inactivated (not shown). The two lines with *P{lacW}* closest to *bw* showed the strongest *trans*-inactivation (inserts marked B and C in Figure 2B). Additionally, s4830 (A in Figure 2B), a line that has a weak eye-color phenotype from a *mini-white* gene inserted 84 kb away from *bw*, shows *trans*-inactivation. However, two lines that contain insertions in *apontic*, a gene which begins 45 kb from *bw*, show contrasting results. The insert (D in Figure 2B) that is in the 5′ regulatory region shows no *trans*-inactivation. Interestingly, an insert in the beginning of the transcribed region (E in Figure 2B) shows very weak, nonvariegated, *trans*-inactivation even though it is actually 463 bp farther away from the bw^D insertion site. Therefore, while distance has an effect on the degree of *trans*-inactivation, there are also local effects of the insertion site.

There is an interesting contrast between the *P{lacW}* insertions at *chrw* (4.7 kb proximal) and *Dcp-1* (15.3 kb distal). While the *P{lacW}/+* flies of both of these lines have approximately the same level of expression, *P{lacW}Dcp-1* is less *trans*-inactivated by two criteria. The background for the highly pigmented spots is quite colorless in the *P{lacW}chrw/*bw^D flies while the background is distinctly yellow in *P{lacW}Dcp-1/*bw^D eyes (Figure 3). Additionally, while both lines show the salt and pepper variegation typical of bw^D *trans*-inactivation, *P{lacW}Dcp-1* has more spots. To quantify this increase in spot number, photos were taken of fly eyes and the spots in a constant area and location (~60% of the eye surface) in each eye were counted. *P{lacW}chrw/*bw^D flies averaged 10.4 spots ($n = 8$, SD = 3.0) and *P{lacW}Dcp-1/*bw^D flies averaged 26.3 spots ($n = 6$, SD = 5.1).

Some endogenous enhancers in the 59E region are insensitive to bw^D *in trans*: In addition to the *mini-white* gene, *P{lacW}* contains a *lacZ* gene coding for β-galactosidase and driven by the weak *P*-element promoter (Figure 2A). This promoter is very sensitive to the presence of enhancers near the site of insertion. Therefore, the expression from this promoter can reflect aspects of the temporal and spatial regulation of genes near the insertion site and has thus been called an enhancer trap (BIER *et al.* 1989). A second transposon used in this study, *P{PZ}*, also contains an enhancer trap (Figure 2A). We obtained a number of fly lines that had been mapped to the 59DEF region from the *P{PZ}* vital locus collection (SPRADLING *et al.* 1999) and tested both these and the *P{lacW}* lines for β-galactosidase activity in young (0–24 hr) or aged (7–10 day) females. We then selected the five lines that showed expression in adult flies. These are the four *P{PZ}* inserts in Table 1 as well as the *P{lacW}chrw* insert. We crossed these flies to Canton-S and to bw^D flies and compared β-galactosidase activity in *P/+* flies to *P/*bw^D flies. A solution enzyme assay was used to determine β-galactosidase activity in extracts from adult flies. Both aged and young males and females were tested in triplicate. In no case did we see any significant difference between the bw^D and wild-type flies (data not shown).

This lack of *trans*-inactivation of the *lacZ* gene in adults contrasted with the *trans*-inactivation of *mini-white* in the same region. We speculated that this may be due to a dependency of silencing on developmental timing, as has been suggested by other researchers (LU *et al.* 1998). Therefore, we examined β-galactosidase activity in wandering late third instar larvae of the five *P{lacW}* shown in Figure 1, four of which showed *trans*-inactivation of

mini-white. Larvae were dissected and stained for β-galactosidase activity. s4830 showed no staining above that observed for bw^D and was not further analyzed. The insertions in *Dcp-1* and *apt* showed adequate staining in either CNS or eye-antennal imaginal discs, and *chrw* showed weak, variable staining in brains. Larvae of these four lines that were *P{lacW}/+* were compared to larvae *P{lacW}*/bw^D at 3 and 18 hr poststaining. Three larvae from each genotype were examined. In no case was *trans*-inactivation seen in the eye-antennal discs or CNS (data not shown).

More evidence for the insensitivity of endogenous regulatory elements to bw^D comes from the fact that we can easily produce flies that contain these lethal *P*-element insertions heterozygous with bw^D. This implies that bw^D is not inactivating the corresponding genes *in cis*. Decreased viability of flies has often been used to assess the variegated inactivation of essential genes (Hearn *et al.* 1991; Howe *et al.* 1995; Martin-Morris *et al.* 1997). As with other PEV alleles of essential genes, one may suspect that *cis*-spreading from bw^D would inactivate the genes that are inactivated by the *P*-element insertions on the homolog and produce a fly with decreased viability. All of the *P{lacW}/CyO* lines in Figure 3 and *P{lacW}k09931/CyO* were crossed to bw^D flies and the numbers of wild-type and Cy progeny scored. The lethality of all these lines, except k09931, had been verified by the *P*-element disruption project as due to the insertion of the *P* element (see materials and methods; Spradling *et al.* 1999; and BDGP). Males from each of the various *P{lacW}* were mated to five bw^D females and removed from the vial after 6 days. All progeny from a cross were scored (between 128 and 313). Crosses were done in the absence of the *P{*bw^+ *cos}* because it contains flanking sequences that could rescue a lethal phenotype. No loss of viability of the wild-type flies relative to their Cy sibs was observed. While this observation does not directly address the strength of *trans*-inactivation, it is unlikely that *cis*-inactivation from the bw^D heterochromatic insertion would be less than *trans*-inactivation. Assuming that the lethal phenotype is caused by a loss-of-function mutation, we conclude that bw^D has little effect on the linked essential endogenous genes.

Effects of bw^D on the *hsp26* and *hsp70* promoters: To test sensitivity of various promoters to the heterochromatic neighborhood we have taken advantage of a *P*-element replacement technique. This allows for the precise replacement of one *P* element with a different one at a separate site (Sepp and Auld 1999). Using this technique we placed the *P*-element construct *P{hsp-w-hsp26-pt-T}* (Figure 2A) into the *chrw* and *Dcp-1* loci. The replaced element lies in the same orientation as the target element and in most cases no detectable rearrangements were found (Figure 1 and materials and methods). Further studies were done on single lines containing *P{hsp-w-hsp26-pt-T}* at each of the two loci. The chromosome containing the insert in *chrw* is called *P{hsp-w-hsp26-pt-T}*$chrw^{ab28}$ and the one in *Dcp-1* is *P{hsp-w-hsp26-pt-T}Dcp-1*^{mgb48}.

The *P{hsp-w-hsp26-pt-T}* transposon contains two reporter genes oriented as shown in Figure 2A. It contains *white* driven by the *hsp70* promoter and a transcript from a plant gene (designated Pt for plant in Figure 2A) driven by the *hsp26* promoter (Wallrath and Elgin 1995). The *hsp70* promoter has both low-level constitutive expression and heat-shock-inducible expression. The constitutive expression of *hsp70-w* accounts for the almost wild-type level of pigment seen in flies carrying this transposon and a null mutation at the endogenous *white* locus.

Both of these lines were crossed to w^{1118};bw^D flies and compared with flies carrying the same transposon at a position on chromosome 2 distant from 59E (as determined by *in situ* hybridization; see materials and methods). Unlike *mini-white*, *hsp70-w* expression did not vary with insertion position in any of the many euchromatic insertion lines we generated and examined. The results for the insertion at the *chrw* locus (4.6 kb away from the bw^D insertion site) are shown in Figure 4A. There is partial salt and pepper *trans*-inactivation of the *hsp70-w* gene in the posterior section of the eye. The expression increases in the more anterior ommatidia. The silencing is never complete; *i.e.*, the background is not white, but yellow-orange, comparable to that of *mini-white* at *Dcp-1*. Additionally, there is a good amount of variation between individuals of the same genotype (Figure 4C). Pigment assays were performed on $chrw^{ab28}/bw^D$ flies as described in materials and methods and the results are shown in Figure 4D.

Classic *cis*-PEV as well as bw^D *trans*-inactivation can be enhanced by lower temperatures and the loss of the Y chromosome (Spofford 1976; Sass and Henikoff 1998). The lower temperature is thought to increase the stability of multiprotein heterochromatic complexes. Loss of the Y, which is entirely heterochromatic, is thought to free up heterochromatic proteins for deposition at other sites, thereby increasing the heterochromatic nature of the remaining sites. Both temperature and the loss of the Y chromosome enhance the *trans*-inactivation of *P{lacW}*$chrw^{ab28}$ flies. An X/O male is shown in Figure 4, A and B, and the effect of growing the flies at 18° is quite similar. Interestingly, it is only the number of spots that is affected, while the background is still the same yellow-orange color.

In contrast to the effects seen on the $chrw^{ab28}$ insertion, we saw no *trans*-inactivation of *P{hsp-w-hsp26-pt-T}Dcp-1*^{mgb48} (Figure 4D), despite the fact that the *mini-white* insertion at this locus showed very strong *trans*-inactivation (Figure 3). We examined whether *trans*-inactivation would be enhanced using lower temperatures and X/O males, alone and in combination, but still saw no *trans*-inactivation.

The White transport protein is deposited in the pig-

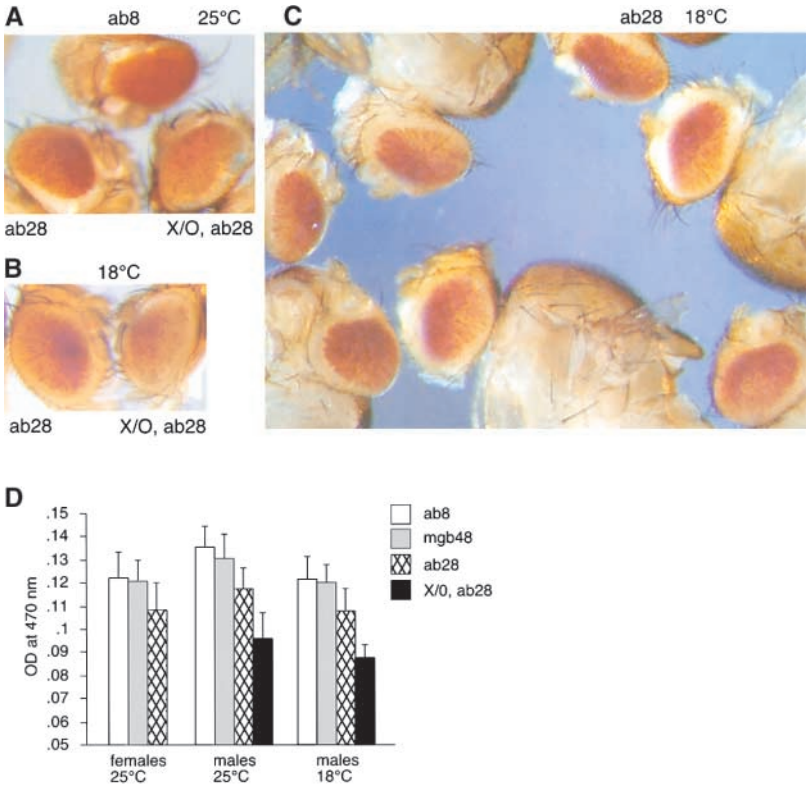

FIGURE 4.—*Trans*-inactivation of *hsp70-white* by bw^D. All flies shown are w^{1118};bw^D/+ and contain *P{hsp-w-hsp26-pt-T}* transgene on chromosome 2 in various locations on the bw^+ chromosome. Flies in the photos are male and aged 1–2 days. Flies were raised and aged at the temperatures indicated in A–D. (A) Chromosome line ab8 contains *P{hsp-w-hsp26-pt-T}* on the second chromosome at a site distant from 59E. ab28 contains *P{hsp-w-hsp26-pt-T}* at the *chrw* locus, 4.7 kb away from the bw^D insertion site. (B) $chrw^{ab28}/bw^D$ flies showing enhancement of *trans*-inactivation by temperature and loss of the Y chromosome. (C) A group of $chrw^{ab28}/bw^D$ flies showing the variation seen in the phenotype. (D) Bar graph showing the absorbance at 470 nm of pigments extracted from fly heads containing various inserts (indicated by shading) of *P{hsp-w-hsp26-pt-T}*/bw^D. mgb48 contains *P{hsp-w-hsp26-pt-T}* in the *Dcp-1* locus 15.4 kb from the bw^D insertion site. $n = 6$; error bars show the 95% confidence interval.

ment cells during mid- to late-pupal stages where it is then responsible for the subsequent transport of pigment precursors. Therefore, the pattern of expression of *white* in the eye is more of an indication of the transcriptional state of the gene at the pupal stage than at larval or adult stages. To evaluate the level of expression in other life stages as well as the amount of transcript from the *hsp26-pt* part of the transgene, we performed Northern blots to determine the level of transcription of the two *P{hsp-w-hsp26-pt-T}* reporter genes in the presence or absence of heterochromatin *in trans*. Total cellular RNA from $Dcp\text{-}1^{mgb48}/+$ and $Dcp\text{-}1^{mgb48}/bw^D$ was examined in 4-day-old female adults grown at 25°. Both heat-shocked and non-heat-shocked flies were examined. No significant decrease in transcript abundance in the bw^D heterozygotes was seen in either the plant transcript or the *white* transcript (data not shown).

When transcript levels were compared in $chrw^{ab28}/+$ and $chrw^{ab28}/bw^D$ flies, *trans*-inactivation was seen only in 4-day-old adults raised at 25° (Figure 5A). *Trans*-inactivation was not seen in either adults or third instar larvae raised at 18°. Indeed, the transcripts from both of the heat-shock promoters appears to be upregulated in larvae (Figure 5B). Both heat-shocked and non-heat-shocked flies were examined, although the transcript in non-heat-shocked flies, especially at 18°, was too low for reliable quantitation.

To determine if a single heat shock at various developmental stages could alter the ability of bw^D to *trans*-inactivate $chrw^{ab28}$, embryos, larvae, or pupae were given single heat shocks at 8, 24, 48, 96, 120, 144, 168, or 192 hr after egg deposition. Thirty adults from each heat-shock time point were scored. Emerging $chrw^{ab28}/bw^D$ adults that had experienced a heat shock during development showed no difference in eye color when compared to $chrw^{ab28}/bw^D$ flies that were not heat-shocked.

***Cis*-inactivation by bw^D:** The relationship between *cis*- and *trans*-inactivation is not well understood. A number of studies have attempted to address this relationship as well as the role of somatic pairing in gene silencing (MARTIN-MORRIS *et al.* 1997; SASS and HENIKOFF 1999). One may suspect that *cis*-inactivation may be substantially stronger than *trans*-inactivation, especially if models concerning the linear crystallization (LU and EISSENBERG 1998; SINGH and HUSKISSON 1998) of heterochromatin along the DNA are valid. To determine if we could detect *cis*-inactivation where we failed to detect *trans*-inactivation, two transposons were recombined onto the bw^D chromosome. These were the *P{hsp-w-hsp26-pt-T}Dcp-1*^{mgb48} insert and the *P{lacW}apt*^{k15608} insert. Neither of these *white* reporters was affected by *cis-PEV*, even when attempts were made to enhance heterochromatic variation by raising these at 18° and examining X/O males. These chromosomes were also tested in the presence of *Df(2R)M41A10* (*Minute of Shultz*), which is a deletion of most of the 2R heterochromatin and enhances bw^D, probably due to a Y-chromosome-like effect (LINDSLEY *et al.* 1960). In all of these tests for *cis*-inactivation at least 100 flies were examined. No change of eye-color phenotype was seen. Therefore, in this system, *cis*-

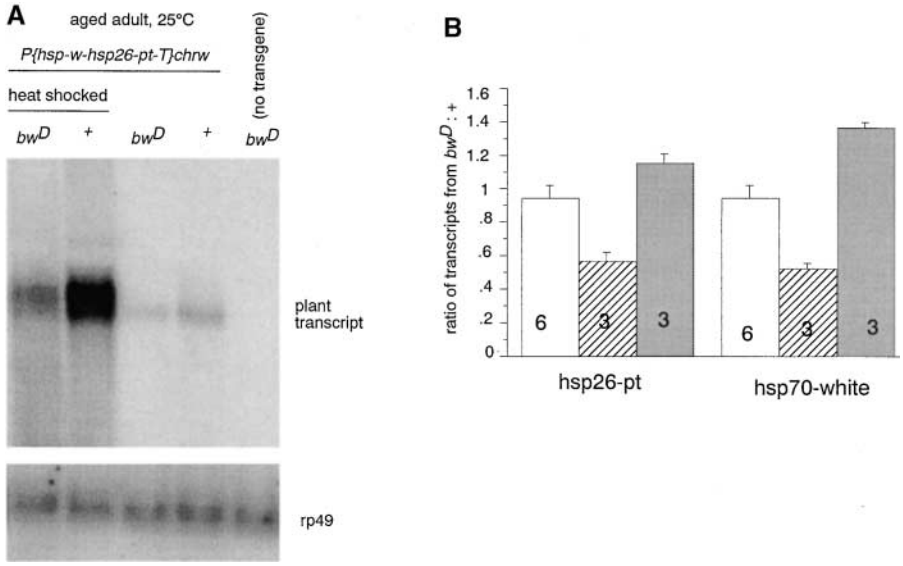

FIGURE 5.—*Trans*-inactivation of transcription from two *hsp* promoters. (A) Northern blot. Total RNA was collected from female adults aged 4 days, fractionated on denaturing formaldehyde agarose gels, Northern blotted, and probed with the plant transcript driven by the *hsp-26* promoter. The blot was then stripped and reprobed with *rp49* to normalize for loading variation. (B) Quantitation of transcript abundance from *hsp26-pt-T* and *hsp70-w*. Bar charts showing the ratio of transcripts from w^{1118}; $P\{hsp\text{-}w\text{-}hsp26\text{-}pt\text{-}T\}\,chrw^{ab28}/bw^{D}$ to *w1118*; $P\{hsp\text{-}w\text{-}hsp26\text{-}pt\text{-}T\}\,chrw^{ab28}/+$ 4-day-old females or wandering female third instar larvae. Three or six (*n* for each data set is shown in box) replicate Northern blots were performed for each genotype. Each lane was normalized using the *rp49* transcript. Quantitation was by phosphorimager. Flies contained a mutation in the endogenous *white* gene that results in the loss of transcription from that locus. Open bars, aged adult, 18°; hatched bars, aged adult, 25°; shaded bars, larva, 18°. Error bars show 1 standard error.

inactivation does not appear to be substantially stronger than *trans*-inactivation.

Heat-shock regulatory regions and nuclear localization: Previous work has shown that bw^D causes the aberrant association of the distal tip of 2R with the centric heterochromatin of that chromosome. The extent of this 59E-2Rh association is correlated with phenotypic expression of *bw* in the adult eye of bw^+/bw^D (CSINK and HENIKOFF 1996). *white* expression in $chrw^{ab28}/bw^D$ flies is many fold higher than *bw* in bw^+/bw^D flies, which is only 2% of the wild-type pigment. Therefore, it was important to determine if the placement of a constitutively expressing regulatory region in the homolog somehow modified the nuclear localization of bw^D. This is key to the interpretation of our results. If the heat-shock promoters on the homolog caused the relocalization of bw^D, then it would be impossible to say if these loci were differentially affected by heterochromatin, since the chromosomal region may not be pulled into the heterochromatic neighborhood to the same extent.

To determine if there was gross change in bw^D heterochromatic association, fluorescence *in situ* hybridization was employed in the same manner as previous studies (CSINK and HENIKOFF 1996). Briefly, probes for a *2Rh*-specific satellite repeat and the 59E region were labeled with different fluors and hybridized to larval CNS nuclei. Distances between the signals were measured (Figure 6A). There was no significant difference in the level of 59E-2Rh association in bw^D flies with or without the $chrw^{ab28}$ insertion at the level of resolution of this study (Figure 6B). Additionally, to test if heat-shock induction of the promoter would change localization, larvae were heat-shocked for 30 min at 37° just prior to dissection. This also did not lead to significant changes in the 59E-2Rh association. However, while this treatment definitely resulted in heat-shock response (see MATERIALS AND METHODS), larvae that were allowed to recover from this treatment and grow to adults showed no phenotypic change in the eye color.

DISCUSSION

Recent work in mammalian systems has begun to identify loci in which transcriptional repression and activation is correlated with association and disassociation with centric heterochromatin (BROWN *et al.* 1997; FRANCASTEL *et al.* 1999; LUNDGREN *et al.* 2000). However, it is difficult to say if transcription is controlling the localization or if the localization is controlling transcription (SCHUBELER *et al.* 2000). In many of the mammalian systems, sequences *in cis* to the tested gene must be modified in order to change transcription and/or localization. Using the ability of bw^D to mislocalize a chromosomal region on the homolog without changing the sequence content of that homolog, we determined the properties of a locus that render it sensitive to changes in interphase nuclear position. We analyzed the ability of bw^D to silence different promoter-reporter constructs at various distances from the *bw* locus. Our system is unique in that we can examine the effects of gross localization on a gene's expression without altering its sequence. We therefore know that nuclear position is influencing a transcription unit that would otherwise be perfectly well expressed.

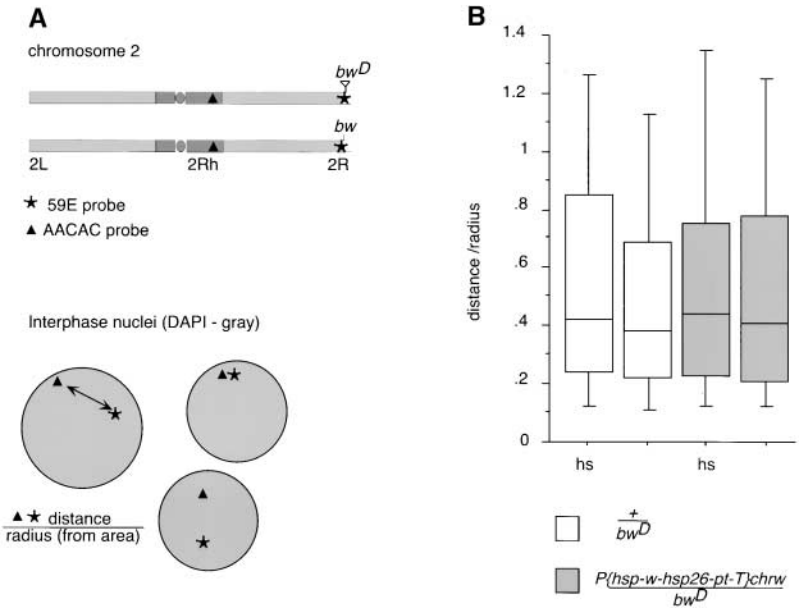

FIGURE 6.—Nuclear localization of chromosomal regions containing *P{hsp-w-hsp26-pt-T}*. (A) Diagram of experimental procedure used to measure the association of 59E with 2Rh. The locations of the two probes on chromosome 2 used in this study are shown at the top. Chromosome 2 is drawn to scale and the heterochromatin is in darker shading. Below is a schematic of an interphase nucleus with the arrow showing the measurement in B and how it was calculated. The top right circle shows the arrangement of probes found when bw^D associates with centric heterochromatin and the bottom right circle shows an unassociated nucleus. Details of the procedure can be found in MATERIALS AND METHODS. (B) Box plots. These show the distance between the *2Rh* probe (AACAC) and the 59E probe divided by the radius of the nucleus. Box plots are calibrated representations of histograms wherein each horizontal line represents the 10th, 25th, 50th (median), 75th, and 90th percentiles. Genotypes and treatments are indicated below and each box plot was derived from at least 90 nuclei. None of the distributions were significantly different from each other as determined using the two-tailed Mann-Whitney *U*-test for nonparametric comparison of two unpaired groups (SOKAL and ROHLF 1981).

Nuclear localization offers a potential mechanism for regulating a large block of contiguous genes by directing the chromosomal region to a specific region within the interphase nucleus. It is possible that a particular sequence within a larger chromosomal region may dictate its nuclear position. This region would then "drag" the contiguous genes with it into a certain nuclear neighborhood. If those contiguous loci were sensitive to their new neighborhood, then the block of chromatin would be influenced in a general manner. Therefore, such localization may be a way to coregulate a set of loci. However, it has not been previously understood how large a region may be influenced by such positional effects, nor how uniform the response of genes in that region might be.

In some examples of classic *cis*-PEV alleles, the distance from the heterochromatic breakpoint to the inactivated gene has been calculated from banding pattern or Southern blotting, but the results have not allowed consistent conclusions to be made because of the many variables associated with such alleles. When comparing the quality and quantity of heterochromatic silencing of different variegating genes and alleles, there are a number of confounding factors. First, there is evidence that different types of heterochromatin are variable in their ability to induce PEV. Additionally, not only could the genes vary in their susceptibility (see below), but the level of inactivation of different genes necessary to detect a phenotype could vary (HOWE *et al.* 1995; TOLCHKOV *et al.* 1998). Also, when considering classic *cis*-PEV, one must consider the placement of the euchromatic breakpoint. The position of the breakpoint will influence not only the distance, but also the inclusion of intervening genes or insulator elements that may influence a distal gene's sensitivity to silencing. Our *mini-white* system is unique in that we do not vary either the heterochromatin inducing the PEV or the reporter gene. Only the position of the reporter gene is altered; intervening DNA is not altered.

The results from the *mini-white* transgene series show that the influence of heterochromatin on this reporter tend to decrease with increasing distance from the heterochromatic insertion site. Two *mini-white* transgenes that are 280 and 460 kb away are not *trans*-inactivated. However, the insertion site also influences the likelihood of inactivation. The *mini-white* insertion in the 5′ regulatory region of the *apt* gene is not *trans*-inactivated, while the transgene insertion that is 463 bp farther away is very slightly *trans*-inactivated. Additionally, even though we see little or no inactivation at *apt*, which is 45 kb distal from the bw^D insertion, s4830 shows almost complete silencing, even though it is a greater distance from *bw* at 84 kb proximal (Figure 3). The s4830 insertion is interesting in that the *mini-white* expression is already quite low and the transgene is located in a region that contains few expressed sequence tag (EST) hits or putative genes (Figure 2B). Perhaps the overall low transcriptional activity of this region renders this region and the inserted transgene more heterochromatic to begin with and therefore even more sensitive to the heterochromatic environment. However, given the variability of the inserts seen above, it will be important to

generate and examine additional *mini-white* insertions in this region.

The differential effects of heterochromatin *in trans* on the *mini-white* insertion is somewhat reminiscent of the work of Howe *et al.* (1995). They examined a *white* transgene near an insertion of heterochromatin and induced flanking, nested deletions of the sequences between the insertion and the heterochromatic block and into the heterochromatic block. Expression of *white* in those derivative lines was not correlated with the size of the deletion or the size of the remaining heterochromatic block. They attributed this to differential effects of the heterochromatin at the breakpoint. In our system, the heterochromatin does not vary and we can say unambiguously that there are both overall distance effects and effects on *mini-white* of the surrounding sequence.

By examining the effect of various promoter-reporter constructs inserted into the same location, we could directly test the idea that the heterochromatic neighborhood is a general, indiscriminate silencer of euchromatic transcription. The various circumstances in which we failed to see *trans*-inactivation indicate that this is not the case. For instance, at the *chrw* and *Dcp-1* loci we examined a total of four different reporters (*mini-white*, *lacZ*, *hsp26-pt*, *hsp70-white*) at each site and the results for the various reporters were different. At *chrw* the small amount of *lacZ* expression was not *trans*-inactivated, while both of the heat-shock promoters were moderately *trans*-inactivated and the *mini-white* gene was strongly *trans*-inactivated. At *Dcp-1* only *mini-white* was *trans*-inactivated. The *mini-white* transgene lacks a number of enhancer elements found in the endogenous locus (Pirrotta 1988). Recently, a study in mammalian cell culture has shown that a gene containing compromised enhancers tends to be localized to heterochromatin and transcriptionally unstable (Francastel *et al.* 1999). A second study has found that a decrease in dosage of an enhancer binding protein causes a gene that is localized to heterochromatin to be less stably expressed (Lundgren *et al.* 2000). Therefore, it follows that the lack of enhancer sequences in *mini-white* most likely renders it highly sensitive to the heterochromatic neighborhood.

The sensitivity of *mini-white* contrasts with the insensitivity of the endogenous genes in the *bw* region that were able to be analyzed by either β-galactosidase activity or lethality. One possibility is that the enhancers they contain confer resistance to the silencing influence of the heterochromatic neighborhood. A second possibility is that they are expressed at a time in the cell cycle or a time in development when the bw^D allele is not associated with centric heterochromatin. This lack of association may render them resistant because either preexisting upregulation is more difficult to silence once the locus gets to the heterochromatic compartment or the genes are expressed only at a time in the cell cycle when bw^D is not associated with heterochromatin. Previous work has determined that bw^D-2Rh associations are broken down in S phase and do not begin to be reformed until at least 5 hr after the start of G1 (Csink and Henikoff 1998). Additionally, in rapidly dividing cells it is possible that there is no association of bw^D with centric heterochromatin. Therefore, if a gene were to be expressed early in G1, it would not be localized to the larger centric heterochromatic neighborhood during the period when its expression is normally on. Such early expression may also set up a chromatin structure that could promote the transcriptional state even if close proximity to heterochromatin is later established. It is worth noting that the one gene in the region known to be sensitive to bw^D, bw^+, is expressed relatively late in pupal development in the differentiated pigment cells (Bhadra *et al.* 1997). Also, the transgenes we analyzed in this study were only those that were inserted into sites that gave rise to a lethal phenotype. This is not a representative sample of the genes in that region (see Figure 2B) or potential insertion sites. Indeed, one may suspect that essential genes would be most likely to be expressed relatively early in development and perhaps insensitive due to some of the above-mentioned reasons.

The *hsp26* and *hsp70* regulatory sequences can be silenced by heterochromatin *in cis* (Henikoff 1981; Wallrath and Elgin 1995). Indeed, the transgene *P{hsp-w-hsp26-pt-T}* is silenced when transposed into centric heterochromatin (Wallrath and Elgin 1995). The ability of bw^D to silence *hsp70* and *hsp26 in trans* is rather weak, but nevertheless apparent at the *chrw* locus for the expression of *hsp70-white* in the eye. This weakness could be due to the more intact nature of the promoter regions of the heat-shock genes relative to those of *mini-white*. Additionally, since the expression from the *hsp-70* promoter has been described as constitutive, it is likely that the *hsp70-white* expression in the pigment cells begins earlier than *mini-white*. The possibility of earlier expression is supported by the observation of a gradation of *trans*-inactivation in the eye, which may reflect the progression of differentiation in the compound eye. Such a gradient is not an intrinsic feature of this transposon construct, as other variegating lines containing this transposon inserted into heterochromatin do not show this gradient (Wallrath and Elgin 1995). During late larval and pupal development of the compound eye the morphogenetic furrow moves from a posterior to an anterior position, so that the posterior-most ommatidia undergo differentiation and exit from the cell cycle ~48 hr before the anterior-most ommatidia. If *hsp70-white* needs to be expressed during a specific time interval for pigmentation, then in some older nuclei bw^D may be associated with the heterochromatic neighborhood during this interval, but in younger nuclei bw^D may not. It would then be more difficult to silence *hsp70-white* expression in the anterior portion of the eye and it would produce the type of gradient we see.

When RNA abundance was used to measure *hsp26*

and *hsp70* inactivation in larvae and adults, we saw *trans*-inactivation only in adults raised at 25°. The lack of *trans*-inactivation in adults raised at 18° is quite strange, especially because the eye phenotype (which is a readout of expression in the midpupae) is enhanced at this temperature. Other workers have suggested that there is a general "relaxation" of heterochromatic silencing upon differentiation (Lu *et al.* 1998) and, while this would account for the lack of inactivation in 18° adults, it cannot explain the contrasting inactivation at 25°.

The results seen for larvae are quite intriguing, as they show an increase of both *hsp*-promoted transcripts in the $chrw^{ab28}/bw^{D}$ heterozygotes. A lack of *trans*-inactivation in larvae was expected, because most of the cells in larvae are polytene. bw^{D}-chromocenter associations are rarely if ever seen in larval salivary gland polytene bw^{D} chromosomes (Talbert *et al.* 1994) and the bw^{+} homolog in bw^{D}/bw^{+} polytene chromosomes appears euchromatic and normally banded (Platero *et al.* 1998). Since the bulk of larval RNA is primarily from polytene cells, it would be surprising if there were *trans*-inactivation. The actual increase in transcript abundance is more difficult to explain. It is possible that there are developmental or tissue-specific upregulators for the *hsp* promoters contained in the bw^{D} line used to produce these larvae. An alternative interpretation is that the large block of heterochromatin next to the transgenes sequesters a certain amount of silencing proteins that would normally be bound to the euchromatin on the homolog. This would result in the upregulation of transcripts on the homolog. Some unique features of polytene heterochromatin make this argument plausible. The bw^{D} insertion is rather highly polytenized when compared to satellite sequences in the chromocenter and the *brown* locus is normally nowhere near the chromocenter because of the Rabl orientation of polytene nuclei. In polytene nuclei, bw^{D} insertion binds a substantial amount of Heterochromatin Protein-1 (Platero *et al.* 1998). Therefore, the upregulation could be thought of as a highly localized Y-chromosome-like effect (Spofford 1976), such that the AAGAG insertion titrates out repressive proteins, resulting in an overall suppression of silencing. On the other hand, one may suppose that a concentration of heterochromatin would lead to a neighborhood silencing effect as described for interphase diploid nuclei. However, such effects may not act across the whole of the polytenized chromosome band, which is relatively large compared to the space of the heterochromatic neighborhood postulated for interphase nuclei. While those copies closest to the homolog might be experiencing silencing, the overall effect of the polytenized heterochromatin *in trans* could be an upregulation of the sequences on the homolog.

No anterior-posterior gradient of eye pigment is seen in the *mini-white*/bw^{D} flies. This may indicate that all of the eye disc nuclei are equally likely to have bw^{D}-*2Rh* associations by the time *mini-white* is expressed. In that case, the expression that is still apparent is a reflection either of the few nuclei that remain unassociated or of a certain amount of expression that is unaffected by heterochromatic neighborhood. A comparison of the inactivation of *mini-white* at *chrw* and *Dcp-1* reveals that the background amount of expression is greater in *Dcp-1*. Assuming that the level of gross association of bw^{D} with centric heterochromatin is unchanged, this indicates that a low level of expression is allowed due to the distance of *mini-white* from the bw^{D} insertion. The higher-expressing spots in both *chrw* and *Dcp-1 mini-white* flies could be due to a few nuclei remaining unassociated. However, one would then expect the number of spots to be equal in the two insertions, since association seems to be unaffected by the presence of an expressing gene (results presented in this article). However, *Dcp-1* has 2.5 times more spots than *chrw*. This increase may indicate that at least some of the high-expressing dots in *Dcp-1* show an ability of slightly more distant loci to overcome the silencing effects of the heterochromatic neighborhood.

Models of PEV have often evoked a linear spreading of higher-order chromatin structure along the chromosome. If these models were true, one would suspect that a locus *in cis* would be much more easily silenced than a locus *in trans*. To test this model we recombined two transgenes that had failed to display *trans*-inactivation onto the bw^{D} chromosome so that heterochromatin could now spread from the insertion site to the transgenes *in cis*. However, we still failed to see any silencing of the reporter genes. These results indicate that silencing *in cis* is not substantially more powerful than silencing *in trans* and suggest that the two processes may be qualitatively similar.

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