Pairing-Dependent Mislocalization of a Drosophila *brown* Gene Reporter to a Heterochromatic Environment

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ABSTRACT

We describe the precise positioning of a reporter gene within heterochromatin where it may be silenced. A transposition of the 59E-60A region into pericentric heterochromatin ensnares distal 59E-60A via somatic pairing. The frequency with which a *brown (bw)* reporter gene in 59E is silenced is influenced by chromosomal configurations. Silencing occurs only when the bw^+ reporter is unpaired due to heterozygosity with a deficiency, where the frequency of bw^+ reporter expression is correlated with the extent of *bw* gene and flanking sequence present. Surprisingly, the frequency of pairing between the transposition in heterochromatin and distal 59E observed cytologically is indistinguishable from the frequency of pairing of homologous chromosomes at 59E in wild-type larval brains, regardless of configuration. Therefore, bringing a susceptible reporter gene into close proximity with heterochromatin does not necessarily affect its expression, but local pairing changes resulting from altered chromosomal configurations can lead to silencing. We also find that an ensnared distal copy of *bw* that is interrupted by a heterochromatic insertion enhances silencing. This demonstrates that *bw* can be simultaneously acted upon by pericentric and distal blocks of heterochromatin.

THERE is an emerging picture of a compartmentalized nucleus in which individual chromosomes not only occupy specific territories but also exhibit particular configurations within that territory (reviewed in Lamond and Earnshaw 1998). This picture pertains primarily to the transcriptionally active euchromatin. However, the transcriptionally inactive and condensed heterochromatin remains poorly differentiated at the cytological level. To address this problem, investigators have used reporter genes juxtaposed to or inserted into heterochromatin as probes of the heterochromatic environment (Weiler and Wakimoto 1995; Martin and Whitelaw 1996; Wallrath 1998). Variable silencing of these reporter genes by heterochromatin establishes the importance of chromosomal context on gene expression.

Recent efforts have extended the connection between chromosomal context of a gene and its expression to include the role of its nuclear position (Wakimoto and Hearn 1990; Csink and Henikoff 1996; Dernburg *et al.* 1996; Brown *et al.* 1997; Park and Boni 1998). In the case of the mutation *brown^Dominant^* (bw^D), a 1–2-Mb insertion of heterochromatin into the *brown* (*bw*) coding region can mislocalize the endogenous bw^+ reporter on a somatically paired homologous chromosome to the pericentric heterochromatin of the same chromosome (Csink and Henikoff 1996; Dernburg *et al.* 1996). A

correlation between the inactivation of the bw^+ gene and its association with centromeric heterochromatin indicates that heterochromatic associations effect gene silencing. Furthermore, genetic observations of a heterochromatic mini-*white* transgene array indicate that heterochromatic associations are responsible for silencing of reporter genes within the mislocalized heterochromatic transgene array (Dorer and Henikoff 1997). Studies in mammalian systems have also shown a correlation between heterochromatin association and gene silencing (Dobie *et al.* 1996; Festenstein *et al.* 1996; Milot *et al.* 1996; Brown *et al.* 1997). In these examples, localization of a gene to a heterochromatic "compartment" is thought to reduce accessibility or exclude factors required for expression resulting in inactivation. In each case, a precisely identified gene sequence associates with a heterochromatic compartment. Unfortunately, the definition of this compartment is so vague as to allow for the possibility that different heterochromatic loci associate with the same reporter in different cells.

In this study, we alleviate the uncertainty about the position of a mislocalized reporter with respect to target heterochromatin responsible for silencing. A reporter gene is mislocalized via somatic pairing to a precise position adjacent to heterochromatic loci that mediate silencing. A heterochromatically embedded segment that includes the *bw* gene behaves as a snare, allowing us to assess the degree of *trans*-inactivation of bw^+ reporter in its normal distal position when it is somatically paired with its heterochromatic homolog. We show that

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in order to be *trans*-inactivated, the reporter must not be paired with its euchromatic homolog. Surprisingly, association between the reporter and the target heterochromatin was seen regardless of whether the gene was *trans*-inactivated, demonstrating that mislocalization is not the only determinant of gene inactivation.

MATERIALS AND METHODS

Fly lines and chromosomes: Flies were reared on standard cornmeal-molasses medium at a controlled temperature of 25°. Table 1 lists the *Drosophila melanogaster* chromosomes used in this study. The bw^{DX7} mutation was generated by an X-ray mutagenesis procedure in the laboratory of Thom Kaufman. Our analysis revealed that this allele should be designated as $Tp(2;2)bw^{DX7}$ and the new order of chromosome *2* given as 21-41|(59E-60A)|41-59E|60A-60F (see results). $Tp(2;2)bw^{DX7}$ is composed of two recombinationally separable components: a duplication of the chromosomal segment 59E-60A transposed to centromeric heterochromatin as well as a deficiency of this segment. For simplicity, we refer to the $Tp(2;2)bw^{DX7}$ chromosome as Tp^{DX7}, the transposed component as Dp^{DX7}, and the deficiency component as Df^{DX7}.

We separated the duplication and deficiency components of $Tp(2;2)bw^{DX7}$ and selected derivatives that contain the duplication component and the wild-type *bw* region. Such $Dp^{DX7}bw^+$ derivatives are formally designated as $Dp(2;2)bw^{DX7}$, bw^+ bw^+ and the new order of chromosome *2* is given as 21-41|59E-60A|41-60F. We also selected the Df^{DX7} [$Df(2R)bw^{DX7}$; 59E-60A] reciprocal recombination products: chromosomes that have a deficiency of the 59E to 60A chromosomal segment.

Cytology: Third instar larval salivary glands were dissected in 1× Ephrussi-Beadle Ringer solution, transferred to 45% acetic acid and allowed to sit for 5–10 min, and then squashed in preparation for polytene chromosome analysis. The procedure used for fluorescent *in situ* hybridization (FISH) was as described (Csink and Henikoff 1996; Marshall *et al.* 1996). For analysis of polytene chromosomes, the probes used were from an 8.4-kb bw^+ genomic clone, an ~40-kb cosmid (cPn-1111) that contains the *bw* gene (Dreesen *et al.* 1988), and cloned genomic DNA derived from the 59E region (P1 DS03480 provided by the Drosophila Genome Project). We also examined mitotic chromosomes from third instar larval neuroblasts that had been prepared for cytology as described (Csink and Henikoff 1996) with the exception that tissue was treated in 0.5% sodium citrate for 10 min prior to fixation and squashing. The satellite sequences AAGAG and AACAC (unique to the centromeric heterochromatin of *2R*) were detected using the appropriate end-labeled oligonucleotides (Csink and Henikoff 1996). These same probes were used in our analysis of somatic pairing and nuclear position in interphase nuclei of third instar larval neuroblasts. In addition, cloned genomic DNA from the 23C region (P1 DS00906 provided by the Drosophila Genome Project) was used as a control for the procedure. Image accumulation and distance measurements were performed as in a previous study utilizing $bw^{Dominant}$ (Csink and Henikoff 1998).

Genetic analysis of bw^{DX7}: When examining the phenotype of heterozygous individuals carrying specific combinations of chromosomes, separate crosses were established to look at paternal as well as maternal inheritance of each chromosome. The parental source of the chromosome had no effect on the phenotype (data not shown). In our analysis of the phenotypic consequence of increased Heterochromatin Protein 1 (HP1), we used a heat-shock-inducible HP1 transgene (HSHP1,83C) and followed a regimen of daily 1-hr heat shocks at 37° through-
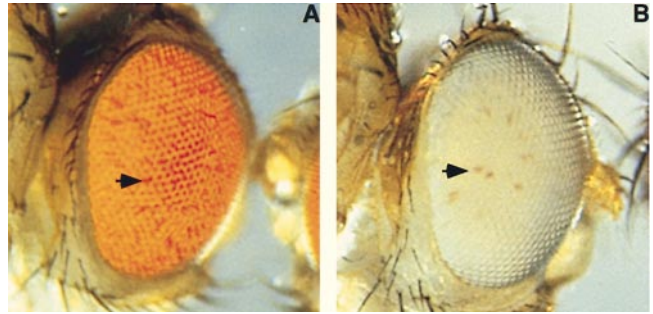

Figure 1.—The bw^{DX7} mutation exhibits atypically weak *trans*-inactivation. (A) Eye from a bw^{DX7}/bw^+;*st* individual showing weak *trans*-inactivation. Arrow indicates a bw^-;st^- ommatidium, which appears darker because absence of pigment causes an interruption in the densely packed array of pigmented cells. (B) Eye from a bw^{DX7}/bw^1;*st* individual showing strong *cis*-inactivation (*i.e.*, silencing of the bw^+ gene present on the bw^{DX7} chromosome). Arrow indicates a bw^+;st^- ommatidium; phenotypically bw^-;st^- ommatidia are white.

out larval and pupal development (Eissenberg *et al.* 1992). The strategy used for design of crosses to assess the phenotypic effect of modifiers of position-effect variegation (PEV) and for scoring eye pigment variegation phenotypes was previously described (Sass and Henikoff 1998).

RESULTS

The bw^{DX7} mutation: The *brown* (*bw*) gene is unusual in that PEV is dominant, displaying silencing both *in cis* and *in trans* (Muller 1932). In general, the strength of the silencing *in trans* mirrors that of silencing *in cis* (Slatis 1955). However, we noticed that the homozygous lethal bw^{DX7} mutation generated in an X-ray mutagenesis screen displayed exceptionally weak silencing of *bw in trans* (Figure 1A), in spite of relatively strong *cis*-silencing (Figure 1B). To elucidate the basis for this atypical behavior, we examined salivary gland polytene chromosomes from individuals carrying the bw^{DX7} chromosome. This revealed a deletion of polytene bands 59E through 60A (Figure 2A), which uncovers the *bw* locus (Dreesen *et al.* 1988). However, the *bw* gene must still be present on chromosome *2*, as individuals heterozygous for the bw^{DX7} chromosome and bw^1 (a null mutation) have bw^+ ommatidia (Figure 1B). Because no other aberration was detected in the euchromatin of chromosome *2*, we suspected that the 59E-60A region may have been transposed to a heterochromatic location on the bw^{DX7} chromosome, where the coalescence and heteropycnotic appearance of the chromocenter impede cytological detection. Fluorescent *in situ* hybridization using probes that contained the *bw* region demonstrated that transposition had occurred: the region was present in the chromocenter of salivary gland polytene chromosomes from individuals carrying the bw^{DX7} chromosome (Figure 2B).

To map the heterochromatic location of the 59E-60A transposition, mitotic chromosomes from third instar
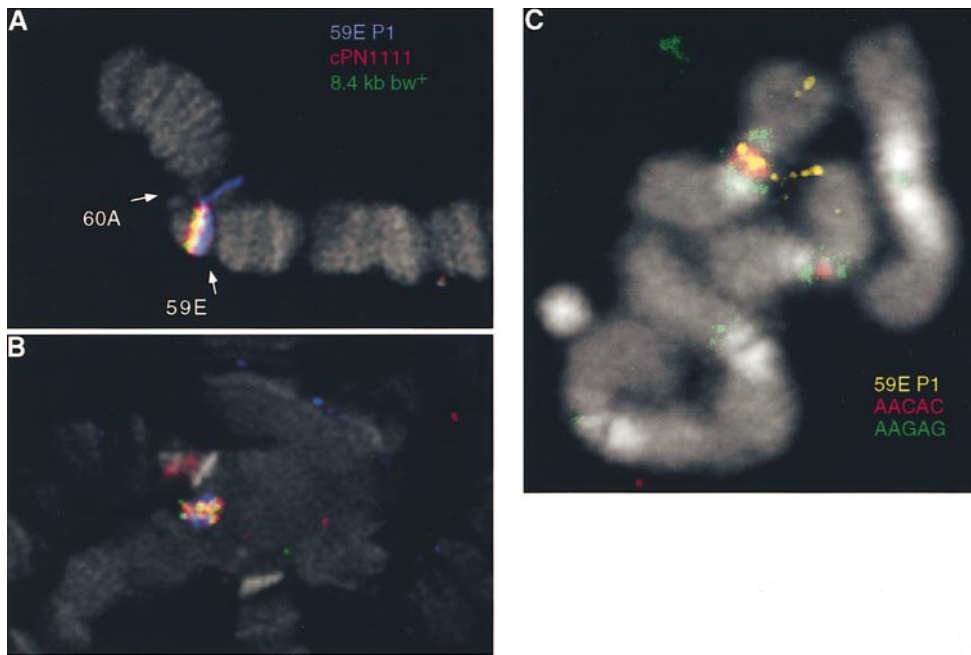
Figure 2.—FISH analysis of the bw^{DX7} transposition. (A) Three different probes from the 59E region decorate the region in wild type, which is looped out due to deficiency of 59E-60A. 59E P1 is an ~80-kb probe proximal to bw, and cPN111 is an ~40-kb probe encompassing bw. (B) The 59E-60A chromosomal segment is present in the chromocenter of the same nucleus. (C) The 59E-60A chromosomal segment (yellow) maps to the pericentric heterochromatin of chromosome 2R in the region of the single AACAC satellite block (red), which is surrounded by AAGAG satellite blocks (green).

larval neuroblasts were examined using FISH. The 59E genomic clone and the satellite sequence (AACAC)$_n$ (found only in centromeric heterochromatin of chromosome 2R) were used as probes. We found that the 59E region was present in the centromeric heterochromatin of 2R (Figure 2C). The relative positions of the 59E and AACAC signals indicate that the site of insertion is either within the AACAC block or very near it. Furthermore, since the 59E probe detected sequences at the centromere as well as the tip of 2R, we were able to confirm in mitotic chromosomes our observation in polytene chromosomes that the proximal euchromatic breakpoint of the transposition is within the region covered by the 59E genomic clone (Figure 2C). We conclude that the bw^{DX7} chromosome is a transposition of region 59E to 60A into 2R heterochromatin (designated Tp^{DX7}; see materials and methods).

Dp^{DX7} silences an unpaired reporter: In the heterozygote Tp^{DX7}/bw^+ there are two copies of the bw region—a copy on the transposed segment, which is embedded in pericentric heterochromatin, and a reporter, which is in its normal distal position on the wild-type homologous chromosome. (Heterozygous combinations used in this study are depicted schematically in Figure 3.) In Drosophila, homologous chromosomes are paired somatically as well as meiotically (Metz 1916). Therefore, in the case of Tp^{DX7}/bw^+, interhomolog pairing of the bw regions would *trans*-inactivate the bw^+ gene on the wild-type chromosome.

We wondered if intrahomolog pairing of bw regions (*i.e.*, between the transposition located in pericentric heterochromatin and the distal reporter on the same chromosome) would also result in *trans*-inactivation. To address this issue, we used recombinant derivatives of

Tp^{DX7} (Table 1). The phenotype of Tp^{DX7}/bw^+ individuals is indistinguishable from that of $Dp^{DX7}bw^+/Df^{DX7}$ (Figure 4A). This indicates that inactivation of a wild-type bw gene mediated by Dp^{DX7} (the chromosomal segment transposed to heterochromatin) occurs similarly whether it is on the same chromosome or on the homologous chromosome. Furthermore, this implies that the region included in Dp^{DX7} is large enough to efficiently pair with homologous sequences at the tip of 2R.

The recombinant derivatives of Tp^{DX7} also revealed that both Dp^{DX7} and Df^{DX7} must be present for *trans*-inactivation to occur: $Dp^{DX7}bw^+/bw^+$ individuals are wild type and do not exhibit bw inactivation (Figure 4B). There are at least two possible reasons for this requirement. Reduction of bw^+ function might be necessary to reveal *trans*-inactivation by Dp^{DX7}. This predicts that any null mutation of bw will expose *trans*-inactivation in combination with $Dp^{DX7}bw^+$. We tested this hypothesis by crossing $Dp^{DX7}bw^+$ to four bw alleles. Flies of all allelic combinations failed to show variegation, including the null mutants bw^1 (Figure 4C) and bw^{2b} (data not shown). Although the molecular defect of three of these alleles is unknown, the bw^1 allele makes normal amounts of a truncated transcript (Dreesen *et al.* 1988). Therefore, the failure to see *trans*-inactivation in genotypes with two copies of the endogenous bw locus is not because an increased amount of bw protein conceals the phenotype, but rather silencing simply does not occur.

An alternative possibility is that a deficiency of or near bw in trans to the reporter might be necessary to expose the reporter, enhancing its susceptibility to pair with the transposed segment and be *trans*-inactivated. To test this, we made individuals heterozygous for $Dp^{DX7}bw^+$ and each of several different deficiencies in this region and
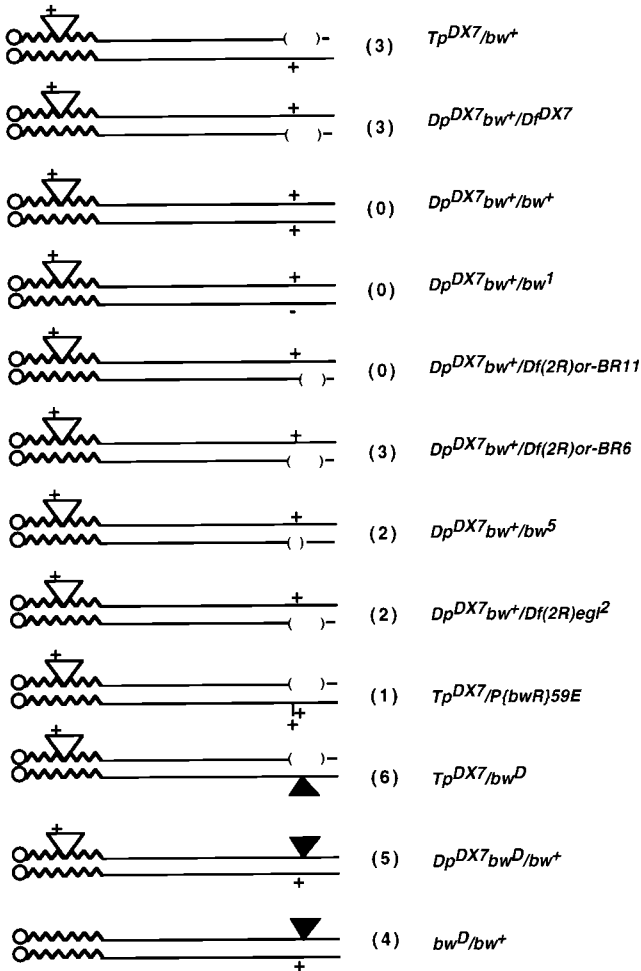
(3) Tp^{DX7}/bw^+

(3) $Dp^{DX7}bw^+/Df^{DX7}$

(0) $Dp^{DX7}bw^+/bw^+$

(0) $Dp^{DX7}bw^+/bw^1$

(0) $Dp^{DX7}bw^+/Df(2R)or\text{-}BR11$

(3) $Dp^{DX7}bw^+/Df(2R)or\text{-}BR6$

(2) $Dp^{DX7}bw^+/bw^5$

(2) $Dp^{DX7}bw^+/Df(2R)egl^2$

(1) $Tp^{DX7}/P\{bwR\}59E$

(6) Tp^{DX7}/bw^D

(5) $Dp^{DX7}bw^D/bw^+$

(4) bw^D/bw^+

Figure 3.—Diagrammatic depiction of heterozygous combinations of *2*R homologs referred to in the text. Heterochromatin is depicted as a wavy line, the centromere as a circle, Dp^{DX7} as an open triangle, bw^D as a filled triangle, deficiencies as parentheses, and a bw^+ transgene as a vertical line. Genotypes are categorized by their degree of silencing (in parentheses), where category 0 appears wild type and category 6 appears almost completely unpigmented. The actual position of the bw^+ gene within Dp^{DX7} component has not been determined.

examined their eye phenotypes. We chose deficiencies that had been generated in a bw^1 null background, allowing us to assess the phenotypic inactivation of the distal bw^+ gene in $Dp^{DX7}bw^+$. Deficiencies of regions either proximal or distal to bw do not show *trans*-inactivation in combination with $Dp^{DX7}bw^+$ (data not shown and Figure 4D, respectively). However, one deficiency, *Df(2R)or-BR6*, did show bw^+ inactivation when heterozygous with $Dp^{DX7}bw^+$ (Figure 4E). This deficiency removes the bw locus, as though deletion of the bw^+ gene itself is necessary for *trans*-inactivation of the reporter.

Df(2R)or-BR6 is comparable in size to Df^{DX7}. The large size of these deficiencies might contribute to *trans*-inactivation by destabilizing somatic pairing in the region. Therefore, we examined the effect of a smaller deficiency of the bw gene on inactivation by Dp^{DX7}. *Df(2R)bw5*

(bw^5) is a deficiency with its proximal breakpoint in the coding region of the bw gene, deleting the 5′ end (A. K. Csink and P. B. Talbert, unpublished data) and extending distally to 59F1, (Dreesen *et al.* 1988). $Dp^{DX7}bw^+/bw^5$ heterozygotes show weak *trans*-inactivation relative to $Dp^{DX7}bw^+/Df^{DX7}$ individuals (Figure 4F). Inactivation was similarly weak in $Dp^{DX7}bw^5/bw^+$ heterozygotes (data not shown). The greater silencing seen with Df^{DX7} than with bw^5 might be caused either by the larger size of the Df^{DX7} deficiency or by the absence of the bw^+ gene in Df^{DX7}.

To determine whether deficiency size or deletion of the bw sequence is important for *trans*-inactivation of the reporter, we used $Df(2R)egl^2$, which removes the same region as Df^{DX7} (59E-60A1, Flybase). The presence of the bw gene in this deficiency cannot be determined phenotypically because it was generated in a bw^1 background. *Trans*-inactivation in $Dp^{DX7}bw^+/Df(2R)egl^2$ individuals is weaker than in $Dp^{DX7}bw^+/Df^{DX7}$ (Figure 4G). Because these deficiencies uncover the same region, we conclude that their different phenotypic effects are attributable to proximal sequences, such as the bw gene, deleted from Df^{DX7} that may still be present in $Df(2R)egl^2$. Consistent with this interpretation, $Df(2R)egl^2$ and bw^5 have breakpoints near bw and display similar *trans*-inactivation when heterozygous with $Dp^{DX7}bw^+$, even though $Df(2R)egl^2$ is about twice the size of bw^5. Larger deletions might enhance *trans*-inactivation by better exposing the reporter as a consequence of freeing up flanking sequences, which would generate a loop. As a result, the exposed region would pair better with the heterochromatically embedded transposition, thus subjecting the reporter to *trans*-inactivation.

If the role of flanking sequence is to interact with the transposition, then a transgene that lacks flanking sequences would escape *trans*-inactivation. Indeed, ectopic transgene insertions are not inactivated at all when heterozygous with Tp^{DX7} (data not shown). Similar observations have been made for other variegating bw mutations (Dreesen *et al.* 1991). Lack of transgene *trans*-inactivation is not due to inherent resistance of ectopic copies of bw^+ to silencing by Dp^{DX7}, because a transgene insertion into 59E1-2, the band that contains *brown*, is *trans*-inactivated (Figure 4H). In this case, the transgene is surrounded by 59E-60A flanking sequences, which can pair with the heterochromatically embedded transposition. Sequences important for *trans*-inactivation have been previously mapped to this transgene segment, close to the 5′ end of the *brown* gene (Dreesen *et al.* 1991; Martin-Morris *et al.* 1993). These observations with transgenes emphasize the crucial role played by sequences flanking *brown* in pairing with the transposition and subjecting the reporter to *trans*-inactivation.

PEV modifiers reveal differences between *cis*- and *trans*-inactivation for bw^{DX7}: To further characterize the bw^{DX7} mutation, we tested the effect of known modifiers of PEV in combination with Tp^{DX7}. *Suvar3-7* and *Suvar2-5*

TABLE 1

Chromosomes used in this study

Name	Description
Tp^{DX7}	$Tp(2;2)bw^{DX7}$; transposition of chromosomal segment 59E to 60A into 2R centromeric heterochromatin
$Dp^{DX7}bw^+$	$Dp(2;2)bw^{DX7}$, bw^+ bw$^+$; derivative contains both the bw^{DX7} duplication and the endogenous 59E to 60A region
Df^{DX7}	$Df(2R)bw^{DX7}$; derivative that carries only the deficiency of 59E to 60A
$Dp^{DX7}bw^5$	$Dp(2;2)bw^{DX7}$, bw^+ bw^5; derivative contains both the bw^{DX7} duplication and the bw^5 mutation
$Dp^{DX7}bw^D$	$Dp(2;2)bw^{DX7}$, bw^+ bw^D; derivative contains both the bw^{DX7} duplication and the bw^D mutation
bw^+	Wild-type chromosome from Amherst strain
bw^D	Null allele of bw caused by a >1-Mb heterochromatic insertion
bw^1	Insertion of ~8 kb into the bw gene creating a null mutation
bw^5	$Df(2R)bw^5$; deficiency from 5′ region of bw to 59F1
bw^{2b}	Null allele of bw
bw^{75}	Hypomorphic allele of bw
bw^{81}	Hypomorphic allele of bw
$Df(2R)Pu$-$D17$	Deficiency that removes polytene bands 57B5 to 58B1-58B2
$Df(2R)or$-$BR11$	Deficiency that removes polytene bands 59F6-59F8 to 60A8-60A16
$Df(2R)or$-$BR6$	Deficiency that removes polytene bands 59D5 to 60B3-60B8
$Df(2R)egl^2$	Deficiency that removes polytene bands 59E-60A1
$P\{bw^+R\}59E$	Insertion of a plasmid containing an 8.4-kb bw genomic fragment into 59E
$YSX \cdot YL, In(1)EN$	Attached X and Y chromosome used in the Y chromosome effect study
$Su(var)2$-5^{02}	Mutation in the gene encoding Heterochromatin Protein 1
$Su(var)3$-7	Mutation in a gene encoding a heterochromatic protein
$E(var)3$-$93D$	Mutation in a gene encoding a protein containing a BTB domain
$Trithorax$-$like$	Mutation in a gene encoding the GAGA protein
$P\{neo^R\}HSHP1.83C$	HP1 transgene under the control of the $Hsp70$ promoter

Except as noted, chromosomes and mutations are described in FlyBase.

are mutations in genes that encode protein components of heterochromatin (Weiler and Wakimoto 1995). Both suppressed *trans*-inactivation in Tp^{DX7}/bw^+ individuals (data not shown). Thus, decreasing the dose of a specific heterochromatic protein relieves the *trans*-inactivation of bw^+ imposed by the bw^{DX7} transposition. In contrast, *Evar3-93D* and *Trithorax-like* failed to enhance (data not shown). These results with bw^{DX7} are similar to those reported previously for the bw^D mutation (Sass and Henikoff 1998).

We increased the levels of HP1 (encoded by *Suvar2-5*) by using an HP1 transgene inducible by heat shock (Eissenberg *et al.* 1992). *Trans*-inactivation in Tp^{DX7}/bw^+ individuals carrying this transgene is strongly enhanced when they are heat shocked throughout development (Figure 5A). Enhancement with increased HP1 was also seen in $Dp^{DX7}bw^5/bw^+$ flies (Figure 5B). We tested whether induction of the transgene might induce variegation in genotypes that showed complete suppression, like $Dp^{DX7}bw^+/bw^+$. There was no effect (data not shown). This extends our previous assertion that inactivation of bw^+ by the bw^{DX7} transposition requires, and is not simply enhanced by, a deficiency of the bw locus.

We also examined chromosomal modifiers of PEV. Y chromosome dosage modifies PEV, with *XXY* females showing suppressed variegation and *X0* males showing enhancement (Spofford 1976). We examined the effects of changing Y dosage on *trans*-inactivation of the

bw^+ reporter. $X\cdot Y/X;Tp^{DX7}/bw^+$ showed suppressed variegation, as expected (Figure 5C). However, the absence of the Y chromosome in $X/0;Tp^{DX7}/bw^+$ males did not result in enhancement, rather it weakly suppressed silencing (Figure 5D). We also examined *Df(2R)M41A10*, which enhances the phenotypes of both *white* (*w*) and *bw* PEV mutations (Lindsley *et al.* 1960). We found that there was no effect of *Df(2R)M41A10* on bw inactivation in $Tp^{DX7}/Df(2R)M41A10$ individuals (data not shown). These findings led us to investigate what effect Y chromosome dosage would have on the *cis*-inactivation of bw^+ within the bw^{DX7} transposition. *Cis*-inactivation can be seen in Tp^{DX7}/bw^1 heterozygotes. Like other bw variegating mutations, *cis*-inactivation of bw^+ in Dp^{DX7} is strong (Figure 1B). Females of the genotype $X\cdot Y/X;Tp^{DX7}/bw^1$ showed suppressed *cis*-inactivation, as expected (Figure 5E). We also found that *X0* males showed enhanced *cis*-inactivation (Figure 5F). Thus, *cis*-inactivation in Dp^{DX7} is enhanced with loss of heterochromatin from the genome, whereas *trans*-inactivation is not. Perhaps only direct changes in the concentration of specific heterochromatin components, such as HP1, can modify *trans*-inactivation in Tp^{DX7}/bw^+.

bw^D enhances silencing in bw^{DX7}: In addition to investigating modification by changes in the amount of heterochromatin in the genome as a whole, we examined the effects of heterochromatin within the bw gene itself using the heterochromatic insertion allele bw^D. $Tp^{DX7}/$
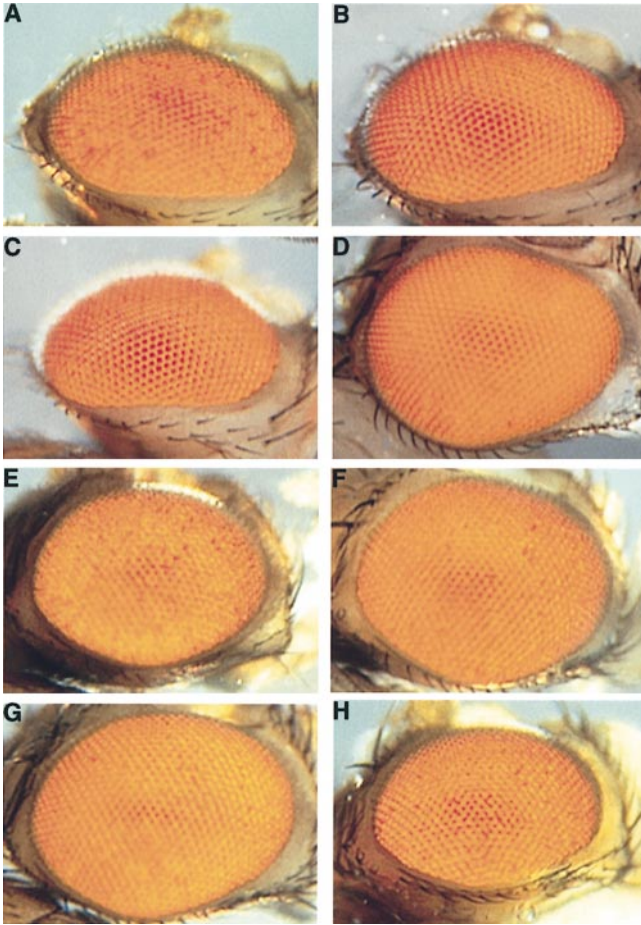
Figure 4.—Analysis of genetic requirements for *trans*-inactivation. (A) Eye from a $Dp^{DX7}bw^+ / Df^{DX7}$ individual. The phenotype is indistinguishable from that of Tp^{DX7}/bw^+ (Figure 1A), showing that *trans*-inactivation is not affected whether pairing between the bw^+ snare and the bw^+ reporter is interchromosomal or intrachromosomal. (B) Eye from a $Dp^{DX7}bw^+ / bw^+$ individual. There is no detectable *trans*-inactivation. (C) Eye from a $Dp^{DX7}bw^+ / bw^1$ individual, demonstrating that activity of the *bw* gene on the homolog is not necessary for a wild-type phenotype. (D) Eye from a $Dp^{DX7}bw^+ / Df(2R)or\text{-}BR11$ individual, demonstrating that deletion of sequences distal to the *bw* gene does not reveal *trans*-inactivation. (E) Eye from a $Dp^{DX7}bw^+ / Df(2R)or\text{-}BR6$ individual, demonstrating that a deletion of comparable size to Df^{DX7}, which also removes the *bw* gene, results in *trans*-inactivation that is indistinguishable from that seen for Tp^{DX7}/bw^+ individuals. *Trans*-inactivation of the bw^+ reporter in $Dp^{DX7}bw^+$ is suppressed when heterozygous to either (F) bw^5 or (G) $Df(2R)egl^2$. (H) Eye from a $Tp^{DX7}/ P\{bw^+R\}59E$ individual, showing *trans*-inactivation of a mislocalized transgene at 59E.

bw^D heterozygotes were more strongly silenced than Tp^{DX7}/bw^1 heterozygotes (data not shown). Such enhancement is opposite from what is expected for addition of extra heterochromatin to the genome and indicates that the large bw^D heterochromatic insertion can directly contribute to silencing of the copy of bw^+ in the transposition. This long-range interaction between a proximal copy of *bw* and distal heterochromatin presumably results from pairing between Dp^{DX7} and se-

quences flanking bw^D. Our interpretation is supported by examination of the phenotypes of other configurations involving bw^{DX7} and bw^D. For instance, $Dp^{DX7}bw^D/ bw^+$ shows stronger *trans*-inactivation than bw^D/bw^+ (735 *vs.* 1346 pigmented ommatidia for 20 individuals of each genotype, $P < 0.0001$), in spite of the fact that it contains an additional copy of bw^+ that must be silenced. To account for this difference, we suppose that bw^D and 2R heterochromatin surrounding bw^{DX7} act additively to enhance silencing of both the reporter and the bw^+ copy in the transposition.

Pairing and long-range associations involving bw^{DX7}: *Trans*-inactivation at the *bw* locus has been proposed to be a consequence of somatic pairing of homologous chromosomes, followed by recruitment of the paired *bw* locus into a heterochromatic compartment of the nucleus, where the *bw* gene cannot be expressed (Csink and Henikoff 1998). Recruitment to a heterochromatic compartment is thought to be mediated by ectopic heterochromatin near the *bw* locus on one of the homologs. *Trans*-inactivation of a bw^+ gene by the bw^D mutation is correlated with its nuclear localization to centromeric heterochromatin (Csink and Henikoff 1996; Dernburg *et al.* 1996). This model for *trans*-inactivation predicts that those cells that continue to express the *bw* gene are those in which *bw* has not been relocated to a heterochromatic compartment.

While the heterochromatic insertion in bw^D appears to relocate the endogenous *bw* gene to a heterochromatic compartment by heterochromatin-mediated associations, Dp^{DX7} relocates the endogenous *bw* region to a compartment by homologous pairing forces. Based on the bw^D model, the very low amount of *trans*-inactivation in Tp^{DX7}/bw^+ individuals might be due to a decrease in the frequency of somatic pairing between the bw^+ reporter on the homolog and the snare-like transposition. This would account for reduced *trans*-inactivation, because the endogenous bw^+ reporter could escape association with a heterochromatic compartment.

To directly test this model, we measured the frequency of somatic pairing between Dp^{DX7} and the distal *bw* locus using FISH with a probe to 59E. There was no significant difference in the frequency of pairing of the *bw* region in third instar larval neuroblasts of Tp^{DX7}/bw^+, $Dp^{DX7}bw^+ / bw^+$, and wild-type individuals (Figure 6A). In all three genotypes somatic pairing was seen as a single hybridization signal in 70–80% of the nuclei. This result is surprising because individuals of the genotype Tp^{DX7}/ bw^+ exhibit *trans*-inactivation, but $Dp^{DX7}bw^+ / bw^+$ individuals are wild type. Importantly, this frequency of somatic pairing is not significantly different from that reported for bw^D/bw^+ individuals (Csink and Henikoff 1998), a genotype in which *bw* is *trans*-inactivated in ∼98% of the pigment cells. That is, somatic pairing is indistinguishably high among genotypes that span a range of *trans*-inactivation from 0 ($Dp^{DX7}bw^+ / bw^+$) to ∼98% (bw^D/ bw^+). We conclude that differences in *trans*-inactivation
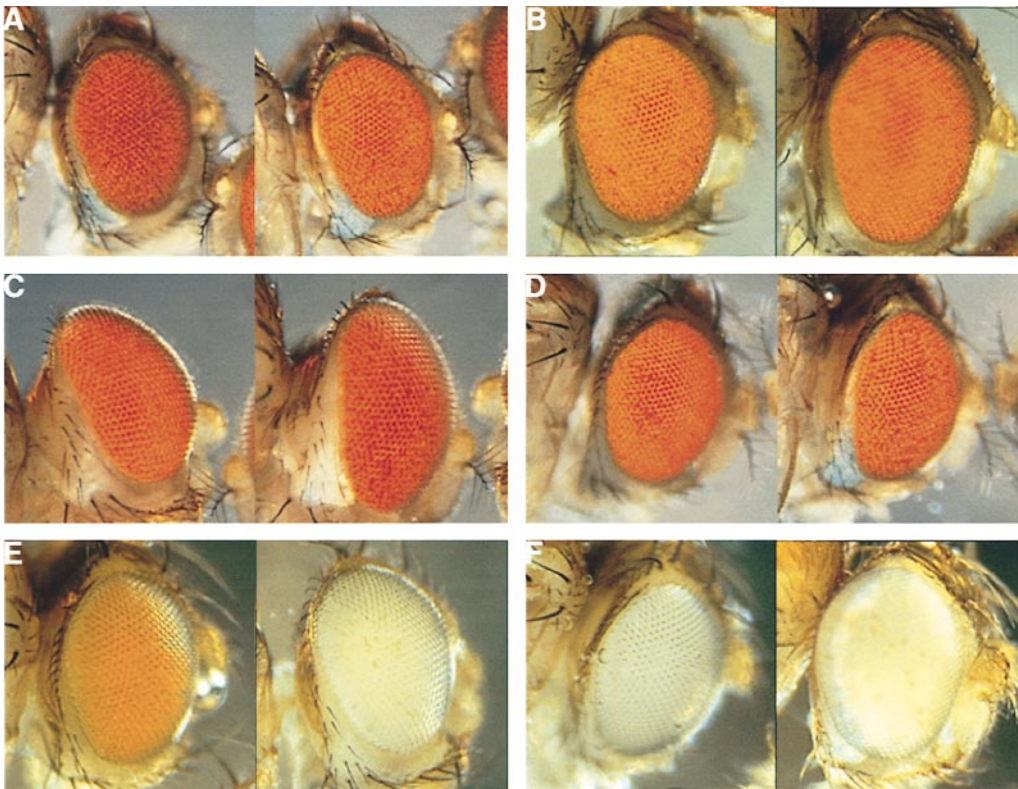
Figure 5.—Analysis of modification of the bw^{DX7} phenotype by modifiers of PEV. Eyes of heat-shocked individuals with (left side in A and B) or without (right side) an HP1 transgene controlled by a heat-shock promoter. Increased HP1 enhances *trans*-inactivation of (A) Tp^{DX7}/bw^+ and (B) $Dp^{DX7}bw^5/bw^+$. (C) Suppression of *trans*-inactivation in Tp^{DX7}/bw^+ by addition of a Y chromosome: eye of *XXY* female (left) and eye of *XX* female (right). (D) Lack of enhancement of *trans*-inactivation in Tp^{DX7}/bw^+ with removal of a Y chromosome: eye of *X0* male (left) and eye of *XY* male (right). (E) Suppression of *cis*-inactivation in Tp^{DX7}/bw^+ by addition of a Y chromosome: eye of *XXY* female (left) and eye of *XX* female (right). (F) Enhancement of *cis*-inactivation in Tp^{DX7}/bw^+ with removal of a Y chromosome: eye of *X0* male (left) and eye of *XY* male (right). Flies are st^+, except for st^- flies shown in E and F.

do not result from differences in the frequency of somatic pairing between the bw^+ reporter on the homolog and the heterochromatically embedded transposition.

Because the frequency of somatic pairing of Dp^{DX7} and the endogenous bw region appeared normal, we considered the possibility that the paired locus may be pulled away from the centric heterochromatin. We examined the relative positions of the bw region and pericentric heterochromatin on interphase chromosomes of third instar larval neuroblasts using FISH. The bw region and centromeric heterochromatin of chromosome *2* were detected with probes to 59E and the satellite sequence AACAC, respectively (Figure 6). The nuclear distance between 59E and AACAC in wild-type, Tp^{DX7}/bw^+, and $Dp^{DX7}bw^+/bw^+$ individuals was determined. These sites were significantly closer together in Dp^{DX7}-bearing cells than in wild type ($P < 0.001$, Figure 6B). Dp^{DX7} relocates the bw locus to centromeric heterochromatin with a mean distance between 59E and AACAC that is comparable to that measured in bw^D-bearing cells (Csink and Henikoff 1998). Furthermore, there is no effect of Df^{DX7} on the 59E-AACAC distance ($P = 0.47$, Figure 6B). Therefore, the differences observed in *trans*-inactivation do not correspond to detectable alterations in interphase configurations.

DISCUSSION

Chromatin associations fall into two general categories: homologous pairing and heterochromatic coalescence. Homologous pairing is the force underlying numerous phenomena, referred to variously as trans-vection (Lewis 1954), *trans*-sensing (Henikoff 1996), and topology effects (Morris *et al.* 1998). Although the mechanism of homologous pairing is not understood, the determinants responsible for these phenomena are often well mapped, typically within gene regulatory regions (Jack and Judd 1979; Geyer *et al.* 1990; Gindhart and Kaufman 1995; Kapoun and Kaufman 1995; Sigrist and Pirrotta 1997). In contrast, determinants for coalescence of heterochromatin have not been localized. For example, the chromocenter of polytene chromosomes involves coalescence of extensive regions, and multiple unknown determinants must be involved. This uncertainty hampers attempts to characterize phenomena that involve heterochromatic coalescence such as nuclear compartmentalization, which is a likely causal factor in PEV (Weiler and Wakimoto 1995). Our study addresses this problem by providing a single site at which compartmentalization and reporter gene silencing can be studied. We utilized a homologous copy of the bw gene region transposed to pericentric heterochromatin

to precisely position a *bw* reporter within heterochromatin. Unexpectedly, we found that when the *bw* region is seen to be mislocalized to heterochromatin in this way, silencing may or may not result.

The expectation that mislocalization in larval brains would be correlated with silencing of an adult eye color gene is based upon cytological and genetic observations with the *bw^D* heterochromatic insertion into the *bw* gene (Henikoff 1996). Heterochromatic associations are seen in multiple tissues and are responsive to chromosomal rearrangements and to suppressors or enhancers of PEV. Moreover, phenomena dependent upon somatic pairing, such as the *zeste-white* effect, are also visualized in the adult eye, even though *white* expression be-

gins during pupal stages. These observations suggest that the nuclear organization seen in larval brains should be indicative of the state of the *bw* gene in the eye. However, we observed differences in levels of *bw* gene silencing despite similar large-scale chromosomal configurations. We conclude that these differences arise from local interactions at the *bw* reporter that occur within the heterochromatic "compartment" of the nucleus.

Our genetic analysis of *bw^{DX7}* provides insight as to what might be happening on a local level to the *bw$^+$* reporter brought into the vicinity of centromeric heterochromatin. Silencing by heterochromatin surrounding the transposition is only revealed when the *bw* reporter lacks a normal pairing partner. When somatic pairing between the *bw* reporter and its pairing partner on the homologous chromosome is reduced by a deficiency, the reporter becomes more strongly silenced by heterochromatin surrounding the transposition. This effect must occur at a local level, because the frequency of somatic pairing detected by FISH is the same as wild-type whether or not the deficiency is present.

We consider two models to explain how local changes in homologous pairing at a *bw* gene in proximity to centromeric heterochromatin can result in differences in silencing. In the euchromatic bubble model, pairing between *Dp^{DX7}* and the endogenous 59E-60A region creates a euchromatic environment that buffers the effects of heterochromatin. When the *bw$^+$* reporter is ensnared in the absence of a third copy of *bw* on the homologous chromosome, the reporter is susceptible to silencing by the surrounding heterochromatic environment. But in
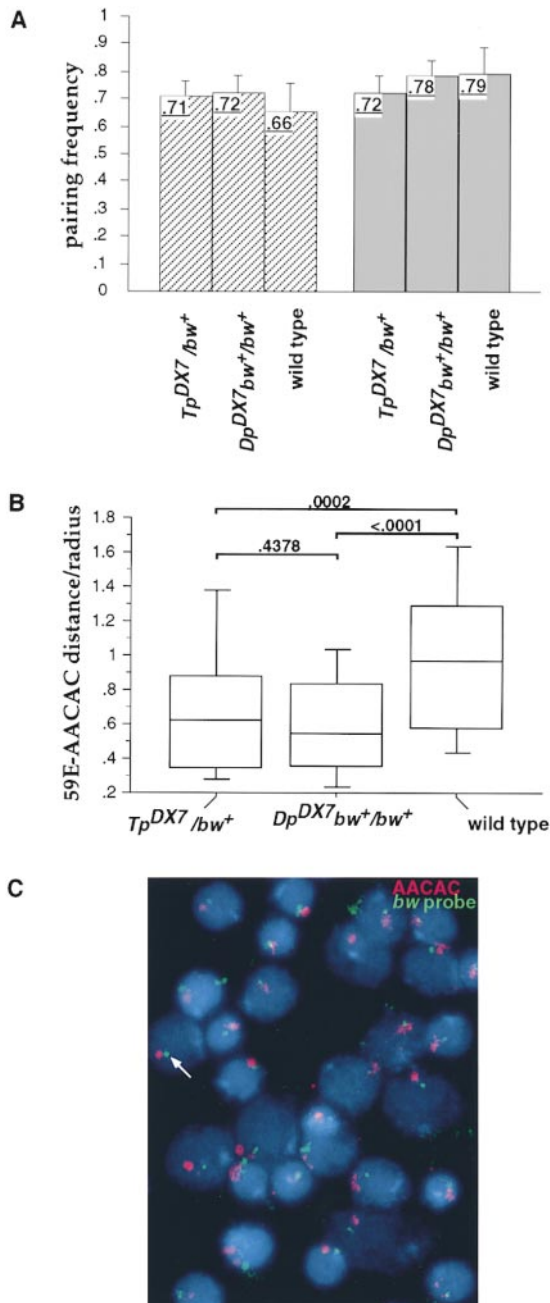

Figure 6.—Cytological analysis of *bw* pairing in larval neuroblasts. (A) Bar graph (with 95% confidence intervals) showing the mean pairing frequency of the *bw* region (light bars) and the 23C control region (dark bars). For each squash examined, the number of nuclei with a single hybridization signal was divided by the number of nuclei with one or two signals, and the mean frequency of single hybridization signals from multiple squashes is shown for each genotype (upper left region of each bar). More than two signals within a nucleus were only rarely detected, with frequencies that did not significantly differ between genotypes. (B) Box plots depicting the distribution of measurements of the nuclear distance between the *bw* region, detected by the 59E P1 probe, and pericentric heterochromatin, detected by the AACAC probe. Each measured distance was divided by the nuclear radius. Measurements were made using the same images collected for analysis of pairing frequency. The bottom of the box shows the 25th percentile, the top shows the 75th percentile, the horizontal line shows the median, and the tails show the 10th (lower) and 90th percentiles. These measurements were subjected to the Mann-Whitney *U*-test and the resulting *P* values are given over brackets. (C) A typical image (from *Dp^{DX7}/bw$^+$*) used to obtain the data for pairing frequency and nuclear distance measurements. The *bw* region probe is in green and a single signal is indicated (arrow); the pericentric AACAC probe is in red.

the presence of a third copy, the increased amount of euchromatin facilitates formation of a "bubble" that provides a buffer against silencing of the reporter. This buffering might result from heightened gene activity. This model is consistent with the observation that increasing the levels of HP1 enhances the phenotype of two-copy individuals, where the weak euchromatic environment would be unstable, but not three-copy individuals, where the stronger euchromatic environment would be stable. However, the euchromatic bubble model is not consistent with the similar phenotypes observed for two deficiencies, bw^5 and $Df(2R)egl^2$, that are very different in size.

In the local unpairing model, the bw^+ reporter escapes *trans*-inactivation when it unpairs from its homolog present in the heterochromatically embedded transposition. Increased unpairing in the vicinity of the reporter would occur when an additional bw gene region that is present on the paired homolog competes for the reporter. This model is supported by the observation that two-copy (Tp^{DX7}/bw^+) individuals exhibit a dilute background level of bw^+ pigmentation (*e.g.*, Figure 4A) that is equivalent to that seen for single-copy flies [*e.g.* $Df(bw)/bw^+$; Slatis 1955; P. Talbert, personal communication]: Local unpairing would result in escape of the bw reporter from *trans*-inactivation, leaving behind the silenced bw^+ copy in the transposition. The euchromatic bubble model does not predict this phenotype, because all copies in a bubble should contribute gene product.

Local unpairing is an attractive model to account for our results because it has been implicated in other examples of transvection and *trans*-inactivation. For instance, it has been suggested that transvection at the *yellow* locus occurs by local unpairing of the promoter, which allows increased access to transcription factors (Morris *et al.* 1999). "Breathing" of the *yellow* promoter in the unpaired state would allow it to capture an enhancer *in trans.* Similarly, breathing of the *brown* promoter in the unpaired state would allow its capture by the transposition, subjecting it to heterochromatic silencing. Previously, a local unpairing model was proposed for *trans*-inactivation of a bw^+ transgene reporter: silencing weakens in proportion to the amount of transgene sequence that is deleted from the paired heterochromatin-linked copy (Dreesen *et al.* 1991). In this case, deletion was hypothesized to cause the formation of an unpaired loop containing the reporter, allowing it to escape silencing by heterochromatin. Deletions of only a few kilobases were effective, revealing that local changes in pairing could modify silencing. In the present study, where a bw^+ reporter was mislocalized to a compartment some tens of megabases away, we nevertheless found that local pairing interactions determine the silencing phenotype.

Local interactions do not entirely account for silencing of the reporter. Previous studies documented a long-range enhancement of silencing by bw^D, interpreted as coalescence of bw^D with *2R* heterochromatin, and coalescence was confirmed in cytological studies (Henikoff 1996). However, it was not clear that bw^D could contribute to silencing beyond its participation in coalescence. Here we have shown that bw^D can indeed contribute to silencing, because silencing was enhanced when this heterochromatic element was ensnared by the transposition. This result is remarkable considering that bw^D enhanced *trans*-inactivation even though the transposition contains an extra copy of bw to be silenced. Thus, silencing appears to result from a combination of large-scale heterochromatic and local pairing interactions.

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