Copy Number and Orientation Determine the Susceptibility of a Gene to Silencing by Nearby Heterochromatin in Drosophila

Joy F. Sabl*,† and Steven Henikoff†,‡

**Department of Zoology, University of Washington, Seattle, Washington 98195 and ‡Howard Hughes Medical Institute, †Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104*

ABSTRACT

The classical phenomenon of position-effect variegation (PEV) is the mosaic expression that occurs when a chromosomal rearrangement moves a euchromatic gene near heterochromatin. A striking feature of this phenomenon is that genes far away from the junction with heterochromatin can be affected, as if the heterochromatic state "spreads." We have investigated classical PEV of a Drosophila *brown* transgene affected by a heterochromatic junction ~60 kb away. PEV was enhanced when the transgene was locally duplicated using *P* transposase. Successive rounds of *P* transposase mutagenesis and phenotypic selection produced a series of PEV alleles with differences in phenotype that depended on transgene copy number and orientation. As for other examples of classical PEV, nearby heterochromatin was required for gene silencing. Modifications of classical PEV by alterations at a single site are unexpected, and these observations contradict models for spreading that invoke propagation of heterochromatin along the chromosome. Rather, our results support a model in which local alterations affect the affinity of a gene region for nearby heterochromatin via homology-based pairing, suggesting an alternative explanation for this 65-year-old phenomenon.

IN higher eukaryotes, heterochromatin differs from euchromatin in both cytological appearance and sequence organization. Heterochromatin appears relatively condensed during interphase, stains differently from euchromatin at metaphase and participates in nonhomologous associations (HEITZ 1929). The sequences that are found in heterochromatin typically consist of blocks of highly or moderately repetitive DNA with a low abundance of genes. An enigmatic feature of heterochromatin is that when euchromatic genes are moved nearby, they show mosaic expression or position-effect variegation (PEV) (reviewed by LEWIS 1950; SPOFFORD 1976; WEILER and WAKIMOTO 1995). PEV is probably not limited to Drosophila, as comparable gene silencing phenomena are seen in mammals (CATTANACH 1974), plants (COCCIOLONE and CONE 1993; MEYER *et al.* 1993) and yeast (ALLSHIRE *et al.* 1994, 1995).

In PEV, heterochromatin appears to spread into euchromatin in a polar manner (DEMEREC and SLIZYNSKA 1937). Spreading can extend through dozens of genes and span megabases of DNA (WEILER and WAKIMOTO 1995). Investigation of this remarkable action at a distance has driven research on PEV for more than 50 years. An early idea was that spreading occurs because homologous pairing interactions between heterochromatic repeats disrupt the structure of the chromosome, affecting nearby genes (EPHRUSSI and SUTTON 1944; HENIKOFF 1994). Later, it was argued that spreading is instead controlled by discrete *cis*-acting elements, including sequences in constitutive heterochromatin that initiate spreading and possibly terminators that limit spreading (EISSENBERG 1989; TARTOF *et al.* 1989; GRIGLIATTI 1991; MOEHRLE and PARO 1994). However, there is no evidence that such elements exist. Moreover, exceptions to the continuity of compacted chromatin (BELYAEVA and ZHIMULEV 1991; BELYAEVA *et al.* 1993) challenge the assumption that spreading occurs by linear propagation of an altered chromatin conformation.

PEV can be used to study heterochromatin in part because *trans*-acting modifiers of PEV encode proteins involved in heterochromatin formation (HENIKOFF 1979; REUTER and WOLFF 1981; MOORE *et al.* 1983; JAMES and ELGIN 1986; LOCKE *et al.* 1988; REUTER *et al.* 1990; GARZINO *et al.* 1992). These include *Suppressor-of-variegation* loci such as *Su(var)205* (GRIGLIATTI 1991), which encodes HP1, a protein component of heterochromatin (JAMES and ELGIN 1986; POWERS and EISSENBERG 1993). A complementary genetic approach would focus on the isolation of *cis*-acting modifiers of PEV located in heterochromatin. However, the large size of heterochromatic blocks and their repetitive sequence composition compromise standard genetic mapping.

Recently, a new genetic approach to the study of heterochromatin was introduced (DORER and HENIKOFF 1994). Repetitive sequences were generated as closely linked duplications of a transposon carrying a mini-*white* eye pigment gene. Repeat arrays of three or more copies showed PEV mutant phenotypes that strengthened with increasing copy number and that were responsive to *trans*-acting modifiers of PEV. This result implies that heterochromatin forms because of the repetitive nature of the sequences found there, not because of the presence of any specific sequences.

Corresponding author: Steven Henikoff, Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Rm. A1-162, 1124 Columbia St., Seattle, WA 98104. E-mail: steveh@howard.fhcrc.org

Although artificial repeat arrays provide a model for heterochromatin formation, they do not address the mechanism of spreading associated with PEV. In part, this is because the same repeat arrays include both the heterochromatin initiation point and the reporters at which heterochromatin acts, so that spreading along the chromosome cannot be assessed. In addition, there are differences between heterochromatin formation in mini-*white* repeat arrays and in classical PEV. For example, classical PEV involves chromosome breaks that juxtapose euchromatin and natural heterochromatin. In the case of mini-*white* repeat arrays, neither chromosome breaks nor natural heterochromatin were involved. Moreover, the mini-*white* reporter gene lacks the normal *white* regulatory elements and therefore is especially sensitive to position effects (KELLUM and SCHEDL 1991; CHUNG *et al.* 1993; ROSEMAN *et al.* 1993).

Here we report an example of classical PEV in which an X-ray-induced chromosome rearrangement has placed natural heterochromatin adjacent to the genomic sequence of a *brown* eye pigment gene with its normal regulatory elements (DREESEN *et al.* 1991; MARTIN-MORRIS *et al.* 1993). Manipulation of this reporter gene using *P* transposase caused striking effects on PEV. These phenotypic changes resulted from local alterations in transposon copy number and orientation. Our findings favor a somatic pairing model over current linear propagation models to explain PEV.

MATERIALS AND METHODS

Fly strains and culture conditions: All *Drosophila melanogaster* stocks were kept at room temperature on standard cornmeal molasses agar medium. Crosses were performed at 25°. Mutants not described below are described by LINDSLEY and ZIMM (1992). Unless otherwise noted, all lines carry *brown*^{Dominant} (*bw*^{D}) and *scarlet* (*st*) giving a white background for easy scoring of changes in expression from *bw*$^{+}$ transposons. *P*[*Δ2,3*](99B) is a third chromosome insertion that encodes *P* transposase (ROBERTSON *et al.* 1988). The line *P*[*bw*$^{+}$]92C is one of 32 previously reported nonvariegating lines (DREESEN *et al.* 1991) produced by injection and subsequent mobilization of *P*-element constructs containing a wild-type genomic copy of the *brown* gene in the pDm24 vector (MISMER and RUBIN 1987); a moderately variegating derivative, *V21*, was produced by X-irradiation (DREESEN *et al.* 1991). *V21* is a translocation that places 2R heterochromatin ~55–70 kb from the *P*[*bw*$^{+}$] transposon (Figure 1A) (J. SABL, unpublished results). *V21* derivatives produced by *P*-transposase mutagenesis are labeled sequentially by phenotype, *i.e.*, (E)nhanced, (R)everted to *V21*-like or (S)uppressed, and isolate number. For example, *V21-E2E1R1* is the product of three rounds of transposase mutagenesis with selection for enhancement (*V21-E2*), further enhancement (*V21-E2E1*), and finally reversion to a *V21*-like phenotype. Transposon orientation is indicated in parentheses after the allele name, *e.g.*, *V21(L)*, *V21-E1(LR)*. Orientations are listed beginning with the most proximal transposon; L indicates that *brown* transcription is directed toward the translocation breakpoint. Transposase-generated derivatives of *V21* are denoted as a group by *V21** and are all marked with *Sb*. *P*[*bw*$^{+}$]92C and its transposase derivatives are denoted as a group by *92C** and are unmarked except by *brown*$^{+}$ expression.

Mutagenesis: Transposase screens were as follows. Males hemizygous for *V21* or *V21** or homozygous for *92C** were crossed to *P*[*Δ2,3*](99B) virgin females (*Δ2,3*). The *92C**/*Δ2,3* and *V21**/*Δ2,3* males were crossed individually or in pairs to five to seven *bw*^{D}; *st* virgin females, and transferred at 4- to 7-day intervals for 2 weeks. For *92C** crosses, male progeny were aged at least 5 days and scored for increased pigment intensity; for *V21** crosses, male progeny were scored for degree of variegation. Putative mutants were crossed to *bw*^{D}; *st* females and maintained by selection. Several concerns influenced the scoring and stocking of putative events. Unwanted events (transposon jumps and mutations in second-site modifiers of PEV) were recovered frequently. Many putative lines were kept for two to four generations to determine linkage before Southern analysis. In the process, some linked lines may have been lost. Additionally, no more than four phenotypically similar events were kept from any one vial. Because of this, frequency estimates are all lower limits. Multiple events from the same vial were scored as independent only if different by Southern analysis. The screen that produced *V21-E2(LL)* and *V21-E3(LR)* used a *bw*$^{+}$; *st*$^{+}$ strain of *Δ2,3*. All other screens were done in a *bw*^{D}; *st* background.

The translocation screen used 2- to 10-day-old males hemizygous for the *V21-E2E1(multi)* translocation chromosome marked with *Sb*. These were X-irradiated (3000 rad), and groups of six were immediately crossed to 30 *bw*^{D}; *st* virgin females. After $3^{1}/_{2}$ days males were removed to insure that only independent events were recovered. Females were transferred after 7 days. Recovery rates of linked suppressors were calculated relative to the total number of *Sb* flies. These lines were designated *V21-(multi)RX*.

Testing with enhancers of PEV: To see if nonvariegating lines could be induced to variegate, females from several *92C** and *V21-(multi)RX* lines were crossed to males carrying second-site enhancers of variegation *E(var)39A*, *E(var)8* and *E(var)66* (LOCKE *et al.* 1988). For second chromosome enhancers, the test class produced was *w*$^{+}$/*w*^{m4} or *w*$^{+}$/*Y*; *Enhancer*, *bw*$^{+}$/*bw*^{D}; *92C** or *V21**, *st*/*st*$^{+}$. For third chromosome enhancers, the enhancer was on the *st*$^{+}$ chromosome, but the test class was otherwise identical. The endogenous *brown*$^{+}$ gene was inactivated by *bw*^{D}. Weak *brown* variegation is generally easier to detect in a *st*$^{+}$ background.

Cytogenetic analysis: Neuroblast chromosomes were prepared essentially as in GATTI *et al.* (1976) without colchicine, then stained in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) 4–8 min, mounted under 90% glycerol, phosphate-buffered saline, 1 mg/ml phenylenediamine (JOHNSON and NOGUERIA-ARAUJO 1981) and photographed with TechPan film (Kodak) through a 100× planapochromatic oil immersion lens under ultraviolet epifluorescent illumination (Nikon UV-1A filter).

Hybridization to polytene chromosomes (SIMON *et al.* 1985) with plasmid, lambda or cosmid DNA was done using randomly primed templates for incorporation of biotinylated dUTP (Bethesda Research Laboratories) and detected with the Detek-1-HRP kit (ENZO Biochemical).

DNA preparation and analysis: *D. melanogaster* genomic DNA was purified by the method of BENDER *et al.* (1983) as modified by J. HIRSH. Restriction digestion and electrophoresis (0.28–1.5% agarose in 1× TAE) were carried out by standard methods (SAMBROOK *et al.* 1989). For standard pulsed field gel electrophoresis, ovary DNA plugs were prepared and digested essentially as described by KARPEN and SPRADLING (1990) for larval disks and brains. Blotting [without neutralization to Biodyne-B (GIBCO)] and radiolabeling with random primers were done by standard methods.

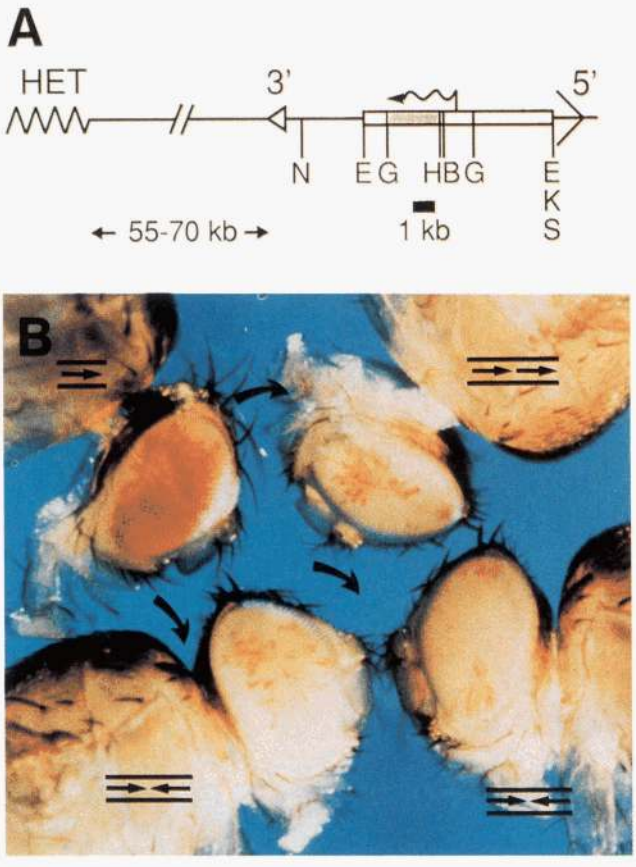

FIGURE 1.—Screens for enhancement of *V21*. (A) The *P*[*bw*$^+$] transposon consists of the pDm24 vector (indicated by 3′ and 5′ transposon ends) containing 8.4 kb of *brown*$^+$ genomic DNA (E-E) including a 3.8-kb fragment (G-G) sufficient for rescue of *brown* (MARTIN-MORRIS *et al.* 1993). Restriction sites are indicated (N, *Nru*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; B, *Bst*EII; K, *Kpn*I; S, *Sma*I). The noncutters *Eco*RV and *Nhe*I were also used. The region used to probe Southern blots is indicated by shading. The *T(2;3)* breakpoint joining 92BC to 2R heterochromatin (zig-zag) was localized using overlapping P1 clones from the Berkeley Drosophila Genome Project, two lambda clones from KATE LOUGHNEY and a cosmid from a library generated by MARK CHAMPE. Based on Southern analysis (with standard and pulsed-field electrophoretic gels) and *in situ* hybridizations, the heterochromatic break maps to P1 clones DS08505 and DS04082, and proximal to DS01965; the transposon insert site maps to the distal end of DS01965 and within DS00599. The wavy arrow represents the extent and 5′-to-3′ orientation of the *brown* transcript. (B) Transposase mutagenesis produces *cis*-acting modifiers of PEV. Here and below, we refer to the various alleles by phenotypic effect, where E refers to enhancers, and (L) and (R) to left and right transgene orientation. *V21(L)* is at upper left; clockwise follow *V21-E2(LL)*, *V21-E3(LR)* and *V21-E1(LR)*. Curved arrows represent $\Delta 2,3$ transposase mutagenesis; small arrows represent copy number and orientation (3′ → 5′) of the transposon.

RESULTS

Selection for enhanced variegation yields transgene duplications: This work began with the fortuitous recovery of an extreme allele derived from a *brown*-variegating translocation line. The original line, referred to as *V21* (Figure 1A), shows patchy pigmentation over about half of the ommatidia (Figure 1B, upper left). This line had been generated by X-ray mutagenesis of flies carrying a *P*[*bw*$^+$] transposon inserted into 92C (*P*[*bw*$^+$]*92C*). The extreme allele, *V21-E1* (previously called *V21*^{e}), shows only scattered spots and patches (Figure 1B, lower left). Both *V21* and *V21-E1* carry the same translocation between chromosome 2R heterochromatin and chromosome *3* broken within 92B (DREESEN *et al.* 1991; data not shown). The juxtaposition of 2R heterochromatin and the transposon causes classical PEV of the *brown*$^+$ gene present on the transposon. Both variegating lines had transposable *brown*$^+$-expressing, and therefore presumably intact, *P* elements, so that the V21-E1 phenotype appeared to result from *cis*-enhancement of the variegating position effect in *V21* (HENIKOFF *et al.* 1993). Structurally, *V21-E1* (like *V21*) is an example of classical PEV, because it was induced by chromosome rearrangement; functionally, its classical nature was confirmed in a screen for *Su(var)*s: 90% of the modifiers that suppressed *V21-E1* also suppressed *white-mottled-4* (TALBERT *et al.* 1994), a prototypic PEV allele (REUTER and SPIERER 1992).

To investigate the basis for this unexpected *cis*-enhancement of the *V21* phenotype, we compared the extreme allele both to its parent allele and to the original nontranslocated line, *P*[*bw*$^+$]*92C*. Southern analysis revealed no differences among the three chromosomes for ~15 kb to either side of the original insertion (data not shown). This suggests that enhancement was not caused by a lesion in nearby sequences. Rather, an alteration involving the *P*[*bw*$^+$] transposon was seen in the extreme line. The entire transposon was duplicated at the original insertion site.

Transposase mutagenesis of *V21* produced three additional linked enhancers from 1260 progeny. All were similar in phenotype to *V21-E1* (Figure 1B), and all were associated with closely linked duplications of *P*[*bw*$^+$] in tandem or reverse orientation as demonstrated by Southern analysis (Figure 2, B and C, and Figure 4C). This perfect correlation between enhancement and duplication (Figure 3) is evidence for a cause-effect relationship.

Enhancement is revertable: Direct proof that duplications are responsible for enhancement requires that removal of the extra copy of *P*[*bw*$^+$] restore the original *V21* phenotype. To this end, we remutagenized two of the duplication lines by exposure to *P* transposase, screened for any phenotypic change, and analyzed the resulting lines for transposon number and structure.

Revertants, which are flies with a moderate-to-dark variegating phenotype approximating that of *V21*, were found at high frequency (see Table 1 and Figure 3). For one line, *V21-E2(LL)*, 10 revertants from 690 flies were characterized. For the other line, *V21-E3(LR)*, three revertants from 70 flies were characterized. All revertant lines had a single copy of the *brown*$^+$ transgene in the same location and orientation as in *V21*. We

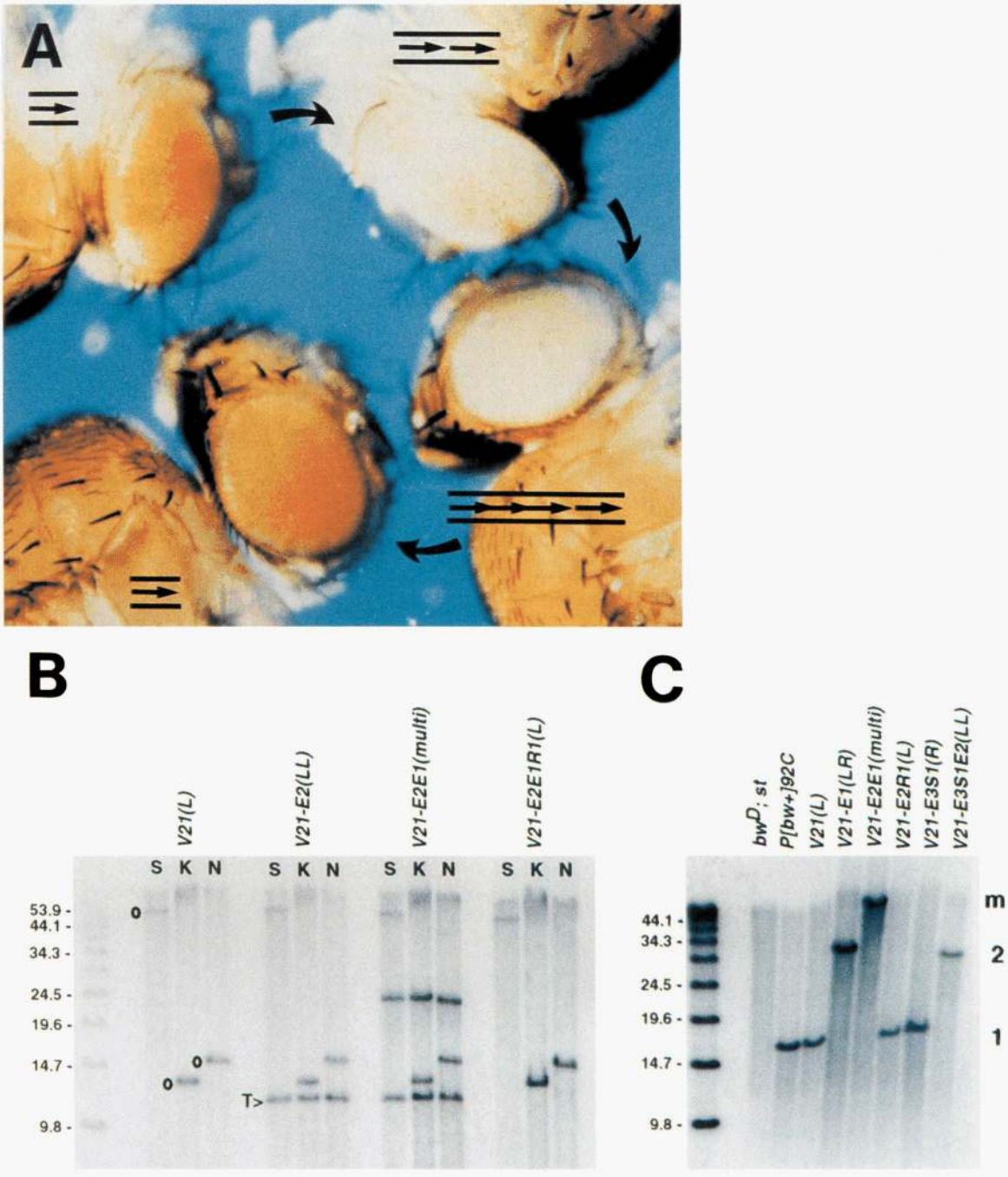

FIGURE 2.—Phenotypes and Southern analysis of duplication-bearing lines. (A) *V21(L)* is at upper left; clockwise follow *V21-E2(LL), V21-E2E1(multi),* and a *V21*-like revertant (abbreviated R). Though three successive rounds of mutagenesis have separated it from *V21(L),* the revertant (lower left) derived from the strongly variegated multi-copy array (lower right) is indistinguishable from the original *V21(L)* line. (B) Single-cutter restriction enzymes *Sma*I (S), *Kpn*I (K) and *Nru*I (N) were used to demonstrate that tandem duplications of a transposon produce a new, approximately transposon-sized band without loss of flanking bands in *V21-E2(LL).* Additional (imperfect) copies of the transposon were also detectable (*multi*); reversion was accompanied by loss of the additional bands in *V21-E2E1R1(L).* Circles mark original bands characteristic of *V21(L)* showing conservation of the 3′ flank (*Sma*I, *Kpn*I) and the 5′ flank (*Nru*I). T marks the band characteristic of a tandem duplication found for all single-cutter enzymes. Unmarked bands reveal extra transposon copies that have size modifications due to deletion in vector sequences. (C) Low-percentage (0.28%) agarose gel analysis using *Eco*RV, which cuts outside of the insert sequences. Nomenclature reflects sequential derivation as illustrated in Figure 3. Size ranges are shown for (1)-copy, (2)-copy and (m)ulti-copy transposons.

conclude that for both (LL) and (LR) alleles, duplications of $P[bw^+]$ cause enhancement of the variegating phenotype.

In addition to revertants, other phenotypic alterations were found. A more extreme derivative of *V21-E2(LL)* had two additional copies of the *brown* transgene at the site (Figure 2). This line, *V21-E2E1(multi),* showed pigment spots only on occasion. *V21-E2E1(multi)* was

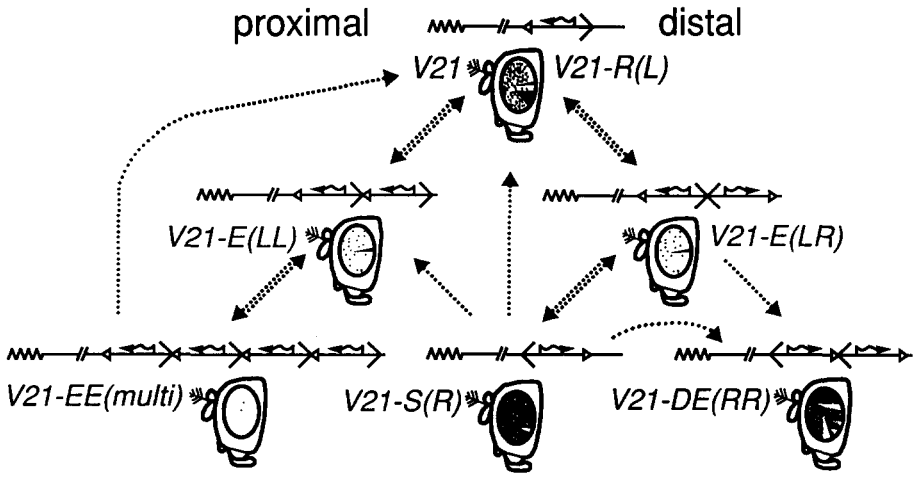

FIGURE 3.—Generation and interconversion of *V21*-derived alleles. Each dashed arrow represents the result of one round of transposase mutagenesis. Typical phenotypes are shown. In addition, flies with fully pigmented eyes (presumed mobilizations) were recovered at every step, demonstrating that at least one transposon and its *brown* gene always remain fully functional. Symbols and nomenclature are as in Figure 1, with S standing for suppressed and DE, for dark-enhanced. (L) elements have 3′ transposon ends oriented toward heterochromatin, and (R) elements are flipped.

subjected to transposase mutagenesis; it, too, reverted to a *V21*-like phenotype or to an intermediate phenotype at high frequency. Of the five derivatives of *V21-E2E1(multi)* that were characterized, all three *V21*-like revertant lines had single intact copies of the *brown* gene, whereas both of the intermediate lines had two to three approximately intact copies of *P*[*bw*$^+$]. These results with *V21-E2E1(multi)* further demonstrate that increased gene copy number leads to decreased frequency of expressing cells.

Orientation changes susceptibility to PEV: The remutagenesis of *V21-E3(LR)* provided the opportunity to examine the effect of PEV on the same transgene inserted in either orientation at the same location. Precise excision of the (R) element from *V21-E3(LR)* results in reversion to a *V21*-like (L) transposon, whereas precise excision of the (L) element in effect reverses the orientation of the transposon at the site. We had expected that the two orientations would lead to the same phenotype. Surprisingly, this was not so. All revertants to the *V21(L)*-like phenotype had lost the (R) copy of the element, reverting to the original (L) orientation. Additionally, flies with nearly wild-type eyes were also retrieved (Figure 4). Upon analysis, these were found to

TABLE 1

Transposase screens of *V21*, *92C* and derived lines

Parent line	Mutant phenotype[a]	No. of independent events[b]	Total flies scored (vials)	Number of *P*[*bw*$^+$] copies[c] (names)
V21	Enhanced var.	4	1260 (31)	2 *(V21-E2, E3, E4)*
V21-E2	Medium var.	>10	690 (12)	1
	Super-enhanced var.	1		4[e] *(V21-E2E1)*
V21-E2E1	Medium var.	>3	570 (16)	1
	Enhanced var.	>2		2–3
V21-E3				
Screen 1	Medium var.	>3[f]	70 (10)	1
	Dark var.	2		1[e] *(V21-E3S1, S2)*
Screen 2	Medium var.	>6[f]	192 (11)	1
	Dark var.	>6[f]		1[e]
	Enhanced dark var.	>2		2[e]
V21-E3S1	Enhanced var.	>3	900 (22)	2
	Enhanced dark var.	2		2[e]
	Medium var.	2		1
V21-E2E1X98	Weak var.	1	~550 (8)	4[d]
92C	Full *brown*$^+$	5	>510 (24)	2

[a] Relative to *V21*.

[b] Number subjected to Southern analysis; phenotypically similar mutants not tested are indicated by >. For all screens, *brown*$^-$ flies were recovered at a rate of 4–30%.

[c] At least one example with complete transposons; other examples have insertions or deletions no larger than 3 kb within transposon copies.

[d] Based on array size for this complex expansion.

[e] Inverted relative to V21.

[f] Numbers are for characterized lines; total frequencies were ~10 times higher.

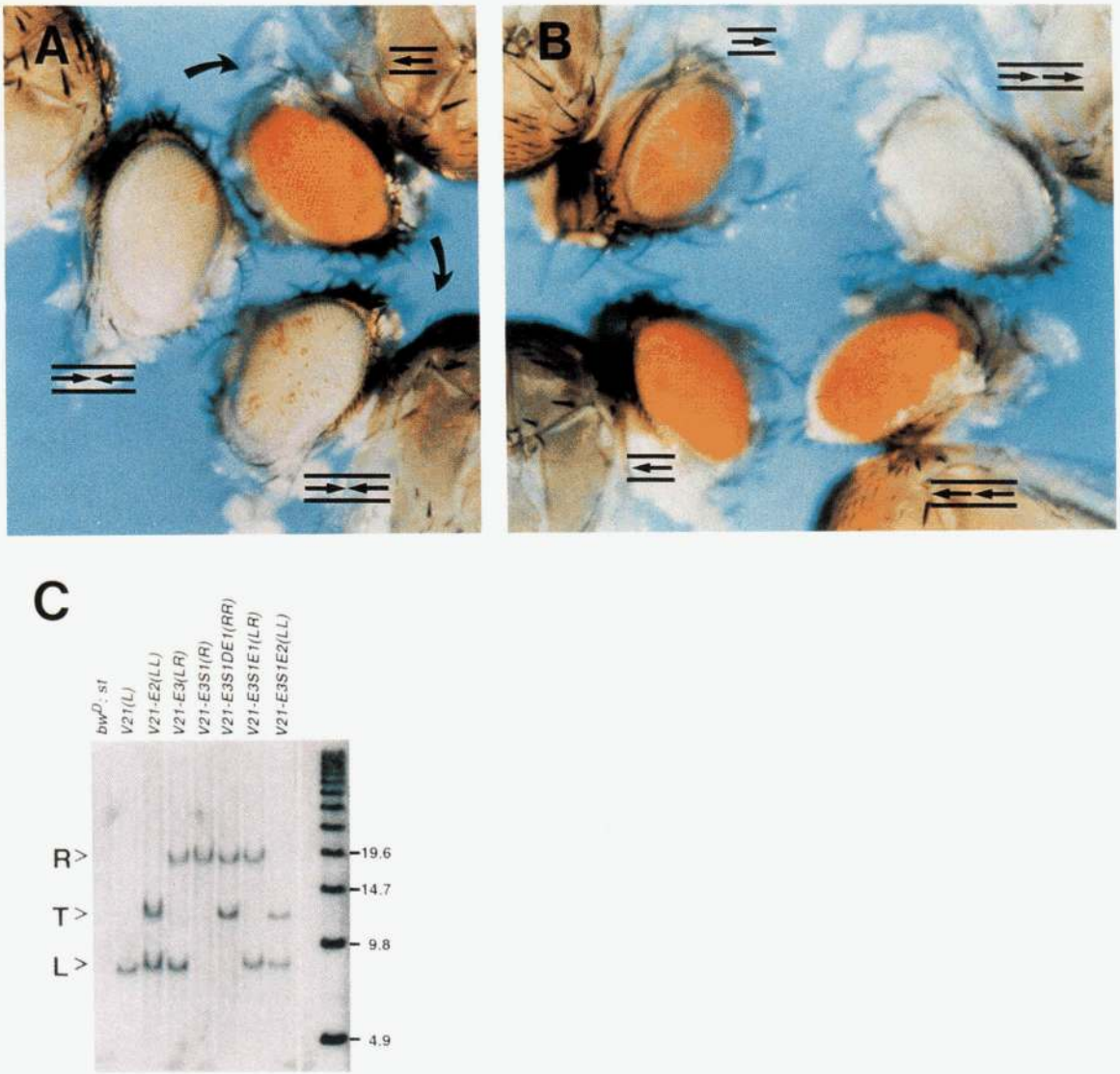

FIGURE 4.—Orientation of *P*[*bw*$^+$] affects PEV. (A) Dark-suppressed lines generated from an (LR) duplication have transposons that are flipped relative to that of *V21(L)*. The dark line *V21E3S1(R)* can give rise to enhanced duplications identical to those produced by *V21(L)*. Clockwise from upper left: *V21-E3(LR), V21-E3S1(R), V21-E3S1E1(LR)*. (B) Tandem duplication of *P*[*bw*$^+$] leads to enhancement of variegation (right side *vs.* left side) and the variegation for the two orientations is strikingly different (top *vs.* bottom). Top: *V21(L), V21-E2(LL)*. Bottom: *V21-E3S1(R), V21-E3S1DE1(RR)*. (C) *Bst*EII digests with a probe from the 3′ end of the *brown* gene (shaded fragment in Figure 1A) showing the characteristic 3′ flanking fragment for single and double transposons in two orientations: L, left; R, right. Tandem (T) duplications are also shown.

be examples of the complementary event: they contained a single transposon in the (R) orientation. Repetition of this screen with intentional selection for the two classes confirmed that phenotype is a perfect predictor of transposon orientation, with six of six lines correctly classified for each orientation (Table 1).

Orientation-dependent phenotypes were also seen for tandem double insert lines. Transposase mutagenesis of *V21-E3S1(R)* produced lines with a weakly enhanced phenotype not previously observed. These were found by Southern analysis to carry two tandem elements in the (RR) orientation (Figure 4, B and C). Although these (RR) lines are enhanced relative to (R) lines, they are strongly suppressed relative to (LL) lines (Figure 4B).

Mutagenesis of *V21-E3S1(R)* also yielded lines with strongly enhanced phenotypes characteristic of (LL) and (LR) lines and lines with moderate phenotypes characteristic of (L) lines (like *V21*). Southern analysis revealed that for 10 lines with unambiguous structures, copy number and orientation were consistent with results of previous mutageneses (Figures 2C, 4C and data not shown). This ability to generate lines with the same phenotypes from different starting points further confirms that the phenotypic effects depend solely on copy number and orientation of *P*[*bw*$^+$].

Variegation requires adjacent heterochromatin: Our observations that PEV strengthens with increasing copy number parallel those reported previously for arrays of mini-*white* transgenes (DORER and HENIKOFF 1994). An

important question is whether arrays of $P[bw^+]$, like arrays of mini-*white*, autonomously form heterochromatin at sites that are distant from heterochromatic regions, so that silencing is intrinsic to the array. In the case of $P[bw^+]$, arrays were produced at the site of a classical PEV allele that juxtaposes euchromatin and heterochromatin via a chromosomal rearrangement. Therefore, our interpretation is that these $P[bw^+]$ arrays primarily enhance classical PEV rather than create new blocks of heterochromatin as in the case of mini-*white* repeat arrays. To address this, we asked whether $P[bw^+]$ duplications could form heterochromatin autonomously at the original 92C site of insertion, which is distant from heterochromatin.

We followed the same protocol used for mini-*white* to expand repeat arrays by transposase mutagenesis of the original (unrearranged) $P[bw^+]$ 92C chromosome. A single $P[bw^+]$ copy at 92C (and most other sites) gives rise to dull red, rather than full red, eyes (DREESEN *et al.* 1991). This mild hypomorphic effect allowed us to screen for duplications at the site by selecting flies with full red eyes. Of these, mutants that showed linkage to 92C were subjected to Southern analysis. This procedure led to the recovery of direct (LL) and reversed (LR) $P[bw^+]$ duplications at a rate of ~1%. These duplication lines did not variegate, nor were any variegating lines recovered. No (RL) derivatives of *92C** (or *V21*) were ever characterized; 3′-to-3′ *P*-transposon duplications are known to occur only rarely (ZHANG and SPRADLING 1993).

Three of the 92C $P[bw^+]$ duplication lines were tested with the *Enhancer-of-variegation* mutations *E(var)66* and *E(var)8* (LOCKE *et al.* 1988); even then, no variegation was observed. Additional rounds of transposase mutagenesis on the tandem lines also produced no variegators, even though >1230 flies were scored. We conclude that $P[bw^+]$ duplications and small repeat arrays at 92C do not have intrinsic silencing ability. Instead, $P[bw^+]$ repeats seem to increase the effect of heterochromatin from the PEV-inducing rearrangement ~55–70 kb away.

In a complementary approach, we asked whether removal of the bulk of centric heterochromatin from the vicinity of the largest $P[bw^+]$ array would fully revert the variegating phenotype. X-ray mutagenesis of *V21-E2E1(multi)* gave suppressed F_1 progeny at a rate of ~0.5%; of these, almost 20% (10 flies) were fully wild type (Figure 5B). Southern analysis revealed that the transgene array was undisturbed in all five full and all four partial revertant lines examined (data not shown). All three of the full revertant lines tested displayed uniform eye pigmentation in the presence of *E(var)66*. Polytene and mitotic cytological analyses on one of these lines, *V21-E2E1X31*, revealed that an inversion had occurred with breaks at 92B and 98D, removing the $P[bw^+]$ array at 92C from the vicinity of heterochromatin. Therefore complete removal of heterochromatin from an extreme variegating array results in full reversion of the phenotype, further suggesting that $P[bw^+]$ repeat arrays act to enhance PEV without forming new heterochromatin.

Another full revertant line insensitive to *E(var)66*, *V21-E2E1X98*, proved to be especially interesting. Linkage analysis showed that the $P[bw^+]$ array was no longer present on a translocation chromosome, confirmed by examination of chromosome *3* in neuroblasts, which showed no visible abnormalities (data not shown). Examination in polytene salivary gland nuclei revealed that this chromosome had "healed," restoring a nearly normal third chromosome but leaving behind a visible block of heterochromatin at 92B5-10 (Figure 5A). In other words, full pigmentation is produced in the *X98* line that retains the full multicopy array, the cytologically visible heterochromatin and the intervening euchromatin of *V21-E2E1*. This result is reminiscent of the many complete revertants of the w^{m4} PEV mutation that were found to involve one break in heterochromatin and another break in euchromatin (TARTOF *et al.* 1984; REUTER *et al.* 1985). Similarly, suppression of a PEV phenotype by movement of a sensitive reporter gene away from pericentric heterochromatin has been seen for *trans*-inactivation of the *brown* gene by the $brown^{Dominant}$ (bw^D) heterochromatic element (TALBERT *et al.* 1994; HENIKOFF *et al.* 1995). There, suppression was found to correlate with a decreased frequency of looping of bw^D into the chromocenter of polytene chromosomes (and *vice versa* for enhancement), lending support to the notion that associations with the heterochromatic compartment of the nucleus underlie PEV.

In addition to the gross rearrangements that modify PEV, more precise changes can have a striking effect on variegation. Recently, it has been shown that PEV is triggered by *P* transposase-induced deletions that bring a single $white^+$ transgene close to an isolated block of 2L heterochromatin (HOWE *et al.* 1995). We wondered whether the trapped heterochromatin in the healed *X98* line could similarly induce PEV. Accordingly, we mutagenized *X98* with *P* transposase and recovered a weakly variegating mutant from ~550 red-eyed progeny (Figure 5B). Polytene chromosome analysis revealed no gross alterations resulting from transposase treatment; however, Southern analysis showed that a deletion had removed intervening sequences, evidently fusing the transgene array to heterochromatin (data not shown). Therefore, proximity of the array to a heterochromatic block influences PEV sensitivity. This inference made on a molecular scale extends previous conclusions based on large-scale cytology (WAKIMOTO and HEARN 1990; EBERL *et al.* 1993; TALBERT *et al.* 1994; HENIKOFF *et al.* 1995).

DISCUSSION

Natural and artificial repeat arrays can interact in forming heterochromatin: We have found that classical

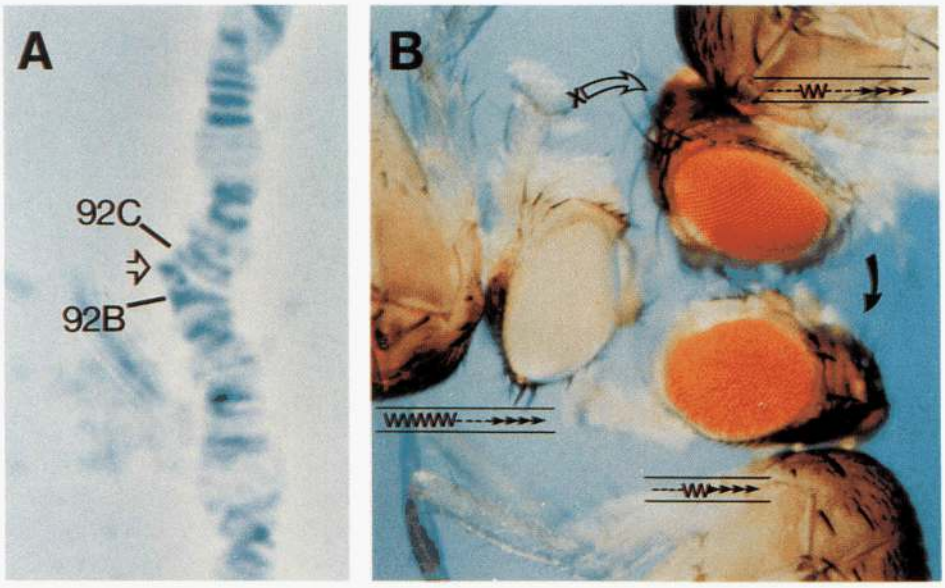

FIGURE 5.—Heterochromatic proximity affects PEV. (A) X-ray-induced healing of *V21-E2E1(multi)* rejoins the tip and the base of the third chromosome, trapping 2R heterochromatin (shown in hemizygote, arrow) between 92B and 92C. The absence of *Eco*RV and *Nhe*I restriction sites associated with this heterochromatic block extends for at least 250 kb. (B) Clockwise from left: phenotypes of *V21-E2E1(multi)*, nonvariegating X-ray revertant; *V21-E2E1X98*, weakly variegating transposase-induced derivative. Based on Southern hybridization analysis (with standard and pulsed-field electrophoretic gels), the weakly variegating line contains a deletion that starts within the array and extends into 92BC euchromatin, ending near or within heterochromatin (data not shown). Zig-zags indicate 2R heterochromatin, dashes 92BC euchromatin and arrowheads transposons. Open curved arrow with X represents X-ray mutagenesis.

PEV, in which a rearrangement causes silencing of a euchromatic gene by juxtaposing it to heterochromatin, can be strikingly altered by local changes at the gene. Tandem and inverted duplications of a *brown* transgene strongly enhance PEV, and longer transgene arrays enhance PEV still more strongly. To an extent, our results with the full *brown* genomic sequence parallel previous findings that transposon repeat arrays become susceptible to heterochromatic inactivation (DORER and HENIKOFF 1994). These effects are unlikely to involve any feature specific to either the sensitive mini-*white* or the fully functional *brown* reporter gene, indicating that heterochromatic silencing of reporter genes is a general property of repeat arrays. Fittingly, *white* and *brown* have been the most intensively studied genes in PEV research, beginning with MULLER's discovery of variegation (MULLER 1930).

Repeat arrays of the *brown* transgene are insufficient to induce heterochromatic effects autonomously, but rather require the presence of nearby natural heterochromatin, as is the case for classical PEV. This finding seems at odds with the autonomy demonstrated by mini-*white* repeat expansions. However, mini-*white* repeats do not appear to be completely autonomous, because an array closer to heterochromatin was more strongly affected than an array farther away (DORER and HENIKOFF 1994), a correlation that has been extended to rearranged derivatives of array-bearing chromosomes (D. DORER, personal communication). Therefore, the difference between *brown* and mini-*white* transgene arrays might be that *brown* arrays are inherently less sensitive to PEV silencing by nearby heterochromatin. Reduced sensitivity would result if insulator elements are associated with $P[bw^+]$ but not with mini-*white*. The presence of insulator elements tightly associated with the *brown* gene is implied by the insensitivity of all $P[bw^+]$ transposons to position effects caused by insertions at various euchromatic sites (DREESEN *et al.* 1991; MARTIN-MORRIS *et al.* 1993). In contrast, mini-*white* lacks insulator elements, as evident from its extreme sensitivity to euchromatic position effects (KELLUM and SCHEDL 1991; CHUNG *et al.* 1993; ROSEMAN *et al.* 1993). Addition of insulator elements protects mini-*white* from euchromatic position effects and provides partial protection from heterochromatic inactivation at sites very close to heterochromatin (ROSEMAN *et al.* 1993). That is, mini-*white* with insulator elements resembles the *brown* genomic segment both in euchromatin and heterochromatin. One known insulator element, scs', corresponds to two DNase I hypersensitive sites (UDVARDY *et al.* 1985) that map exactly to the shared promoter region of two divergently transcribed genes (GLOVER *et al.* 1995). Insulation may be a common feature of Drosophila promoter regions.

At mini-*white* repeat arrays, strong pairing interactions based on sequence identity have been proposed to nucleate heterochromatin formation (DORER and HENIKOFF 1994). Similarly, $P[bw^+]$ arrays would be subject to the same forces and participate in the same kinds of pairing interactions. The *brown* insulator sequences

would merely inhibit later steps in heterochromatin formation, such as the assembly of *Su(var)*-encoded proteins.

Orientation dependence suggests sequence-specific interactions with heterochromatin: An unexpected finding was that reversing the orientation of the transposon carrying the *brown* gene modified the variegating phenotype. Differences were seen both for single copies and for tandem duplications. These differences cannot be explained by the proximity of heterochromatin to the promoter, because the promoter is slightly farther from heterochromatin in the more strongly inactivated lines. Similarly, they cannot be explained by imagining an insulating block specific to either end of the element. For example, a partial block 5′ to the transcription unit could indeed account for the observed differences between (L) and (R): such a block would protect (R) but not (L) from PEV. However, in such a case, both (LL) and (LR) two-copy lines would have the more distal copy protected, and therefore be dark in phenotype, rather than light.

Pairing properties might contribute to some of the phenotypes, explaining why (LR) lines resemble (LL) lines, whereas (RR) lines are darker. For single transposons, flipping from (L) to (R) gives a darker phenotype. This leads to the expectation that (LR) lines should be darker than (LL) lines. However, a counter expectation, based on the phenotypes of mini-*white* repeat arrays, is that pairing within (LR) lines should be stronger than pairing within (LL) lines, so that (LR) lines should be lighter than (LL) lines. We suppose that these conflicting effects cancel one another.

Weaker pairing properties could account for the orientation effect, in which (R) lines are darker than (L) lines and (RR) lines are darker than (LL) lines. It is possible that limited stretches of sequence similarity exist between the transposon and nearby heterochromatic sequences and that occasional pairing occurs. Another possibility is that stretches of chromatin similarity exist, such as similar patterns of nucleosome phasing or similarly ordered DNA-binding proteins, leading to pairing of heterologous sequences. These possibilities cannot be distinguished given our lack of understanding of the mechanism that underlies somatic pairing. In either case, the frequency of pairing would be greater for antiparallel than for parallel orientation, resulting in a higher frequency of *brown* gene inactivation.

Another possible explanation for orientation dependence arises from an unusual feature of the *brown* gene, that heterochromatin can inactivate the gene in *trans*, causing dominant position-effect variegation. This dominant effect might be mediated by a heterochromatin-sensitive transcription factor that makes direct contact with heterochromatic proteins (Dreesen *et al.* 1991; Martin-Morris *et al.* 1993; Martin-Morris and Henikoff 1995). This hypothetical transcription factor would then interact with heterochromatin in a particular topological orientation. However, recent results show that mini-*white*, which lacks transcription factor binding sites, can be *trans*-inactivated (D. Dorer, personal communication), undermining this explanation.

Although other models might be proposed to explain orientation dependence, this effect is not explained by sequence-independent properties of chromatin. That is, simply reversing the orientation of a DNA segment without changing the distance to heterochromatin should not affect the distribution of chromatin proteins that lack sequence specificity. Rather, we suggest that orientation dependence of single and tandem copies is understandable as a consequence of the inherent polarity of DNA sequences. Relevant to this suggestion, bacterial site-specific recombination can show an orientation dependence over distances of >10 kb (Howe and Schumm 1981). Recombination studies *in vitro* suggest that orientation effects do not involve linear propagation or tracking; rather, topology constrains looping interactions between similar sequences (Gellert and Nash 1987).

A pairing-looping model for PEV: Current models for PEV are motivated by the well established polarity of the effect, in which genes close to a heterochromatic break are more strongly affected than genes farther away (reviewed by Spofford 1976). These models propose that the heterochromatic state propagates linearly along the chromosome from sites within constitutive heterochromatin (Zuckerkandl 1974; Eissenberg 1989; Tartof *et al.* 1989; Grigliatti 1991; Moehrle and Paro 1994). Chromosome breaks that join heterochromatin to euchromatin might allow propagation into euchromatin. To account for mosaicism of gene expression, linear propagation must terminate variably either before or after that gene in different cells. In a single cell, the gene is active only if propagation has stopped in the 55–70 kb stretch proximal to the gene. Changes distal to the stopping point should not have any effect, unless it is imagined that propagation can be attracted or repelled from a distance. Yet we find that such distal changes are extremely effective in modifying inactivation of the *brown* gene. For example, *V21-E3S1(R)* has eyes that are nearly wild type with rare mutant patches, whereas *V21-E2E1(multi)* has eyes that are fully mutant except for an occasional eye with one or more wild-type spots, that is, changes distal to the hypothetical propagation stopping points produce phenotypes spanning the full observable range. This contradicts models that require continuous linear propagation of heterochromatin.

Rather than invoking linear propagation or action at a distance to explain our results, we suggest that silencing results from direct contact between heterochromatin and the affected gene. This contact could be mediated by the forces of somatic pairing, an interaction seen most vividly in the salivary gland polytene chromosomes. Polytene chromosomes display both homologous association of chromatids and nonhomologous

association of heterochromatic blocks into a chromocenter. We propose that sequences moved close to heterochromatin can associate directly with the chromocenter or heterochromatic compartment of the nucleus. Orientation dependence seen for single or tandem $P[bw^+]$ transposons suggests that such associations reflect pairing interactions between sequences on either side of the euchromatin/heterochromatin boundary. Increased silencing with increased size of the transposon array would reflect the tendency for repetitive sequences to pair, form recognizable structures (DORER and HENIKOFF 1994), and colocalize with heterochromatin.

Our model can account for the bias in breakpoint distributions and frequencies seen for different variegating genes (SPOFFORD 1976). This bias suggests that the large majority of breaks that place a reporter gene near heterochromatin do not produce a variegating phenotype and so are not selected in a screen. The middle repetitive elements found clustered in heterochromatin and scattered throughout euchromatin could provide the compatibility needed for mediating PEV (Figure 6). When a chromosomal rearrangement moves a gene close to heterochromatin, then the net effect of pairing between any element near the gene and similar sequences clustered in heterochromatin will be to drag the gene close to the heterochromatic compartment, causing inactivation. This possibility also explains the surprising presence of middle repetitive heterochromatic sequences at PEV breakpoints (TARTOF *et al.* 1984), rather than the more abundant simple sequence DNA in heterochromatin (LOHE *et al.* 1993). Unlike middle repetitive elements, simple sequence satellites are rare or absent in Drosophila euchromatin and so cannot mediate contact between euchromatic sequences and the chromocenter. The model is also consistent with the lack of correspondence between the quantity of heterochromatin at a single breakpoint location and the severity of PEV (HOWE *et al.* 1995), because specific repetitive sequences in heterochromatin that mediate looping presumably lie at varying distances from compatible euchromatic sequences in different lines. The model is economical, in that it proposes a single underlying mechanism, somatic pairing, for both heterochromatin formation and PEV.

Our model also can rationalize the binding pattern of antibody to the *Su(var)*-encoded HP1 protein. Anti-HP1 binds not only to the polytene chromocenter, but also to euchromatic sites, such as the banded regions of the fourth chromosome (JAMES and ELGIN 1986; POWERS and EISSENBERG 1993). Interestingly, this pattern is also seen for known middle repetitive elements (MIKLOS *et al.* 1988). An HP1-containing protein complex might bind specifically to somatically paired structures that form at locally repetitive sequences (DORER and HENIKOFF 1994), and this would favor looping associations with similar sequences nearby. Comparable local looping of DNA-protein complexes has been proposed to explain the spread of silencing by *Polycomb*-group proteins (PIRROTTA and RASTELLI 1994). In this way, unrelated gene silencing phenomena mediated by different *trans*-acting factors with different specificities might nevertheless involve analogous protein-protein interactions (PARO 1990).

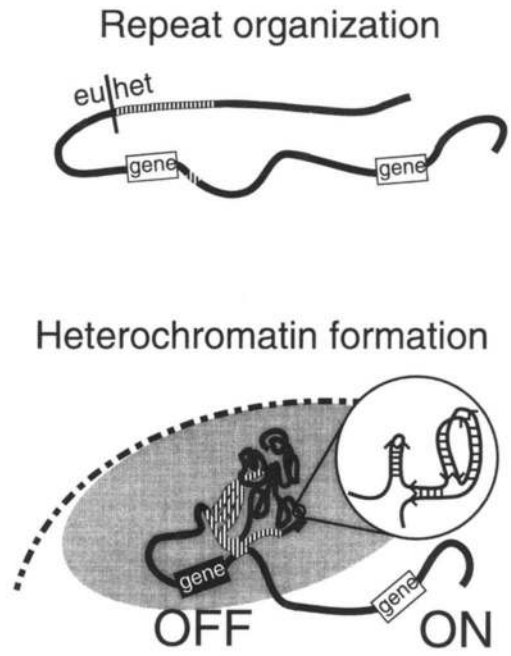

FIGURE 6.—A model for PEV. The repeat organization of a chromosome is shown in the vicinity of a classical variegating rearrangement breakpoint. The solid line represents single-copy DNA in euchromatin (eu) and the striped and shaded lines represent two different families of repetitive sequences, present both in large blocks in heterochromatin (het) and as dispersed middle repetitive elements in euchromatin. Heterochromatin forms as a result of local pairing between homologous double-stranded DNA sequences forming hairpin, loop and more complex structures (magnified), which participate in the formation of a chromocenter (gray oval) on the nuclear envelope (dotted line). Silencing of a gene by PEV can occur when a dispersed repeat nearby pairs with a homologous sequence in a block, sequestering the gene into a heterochromatic environment.

In conclusion, we have shown that PEV is strikingly modified by purely local effects that can be understood in terms of somatic pairing forces acting on repeats to form heterochromatin (EPHRUSSI and SUTTON 1944). Our results support the view that pairing associations organize the nuclear compartment and regulate the effects of chromosomal rearrangements on gene activity (DEVLIN *et al.* 1990).

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