Transgene Repeat Arrays Interact With Distant Heterochromatin and Cause Silencing in *cis* and *trans*

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ABSTRACT

Tandem repeats of Drosophila transgenes can cause heterochromatic variegation for transgene expression in a copy-number and orientation-dependent manner. Here, we demonstrate different ways in which these transgene repeat arrays interact with other sequences at a distance, displaying properties identical to those of a naturally occurring block of interstitial heterochromatin. Arrays consisting of tandemly repeated *white* transgenes are strongly affected by proximity to constitutive heterochromatin. Moving an array closer to heterochromatin enhanced variegation, and enhancement was reverted by recombination of the array onto a normal sequence chromosome. Rearrangements that lack the array enhanced variegation of *white* on a homologue bearing the array. Therefore, silencing of *white* genes within a repeat array depends on its distance from heterochromatin of the same chromosome or of its paired homologue. In addition, *white* transgene arrays cause variegation of a nearby gene in *cis*, a hallmark of classical position-effect variegation. Such spreading of heterochromatic silencing correlates with array size. Finally, *white* transgene arrays cause pairing-dependent silencing of a non-variegating *white* insertion at the homologous position.

THE expression of a eukaryotic gene can be influenced by its position within a chromosome. Position effects on gene expression are often revealed by chromosomal rearrangements that place genes within new sequence contexts or by differences in expression patterns of transgenes integrated at different chromosomal sites. Some position effects are caused by proximity of the affected gene to enhancer or silencer sequences, whereas others may be attributed more generally to the degree of local chromatin compaction. The chromatin of most eukaryotic cells is divided into two types, euchromatin and heterochromatin, that are distinguished cytologically by their differences in compaction through the cell cycle. These two types of chromatin generally occupy distinct regions of chromosomes, with the heterochromatin being primarily pericentric. Euchromatic genes are silenced by rearrangements that place them within or near heterochromatin, a phenomenon known as position-effect variegation (PEV). These rearrangements produce a variable mosaic pattern of gene inactivation in which fully expressing cells are found adjacent to cells that fail to express the relocated gene.

A notable feature of PEV is that an affected gene may lie at a considerable distance from a rearrangement breakpoint (SPOFFORD 1976). The ability of heterochromatin to cause gene silencing over a distance has often been interpreted as the linear propagation of a condensed chromatin state from the rearrangement breakpoint into neighboring euchromatic sequences. In this model, DNA sequences within heterochromatin serve as nucleation sites for the formation of a complex of heterochromatin-specific proteins and the propagation of this complex along the chromosome, silencing the euchromatic genes that it incorporates (TARTOF *et al.* 1989). This model can account for the observed polarity of gene silencing: genes closer to a rearrangement breakpoint are more frequently affected than more distal genes and appear to be inactivated in any cell in which more distal genes are silenced (DEMEREC and SLIZYNSKA 1937; SCHULTZ 1939). However, more recent data suggest that the linear propagation model is inadequate to account for all of the properties of PEV (SABL and HENIKOFF 1996).

An alternative model is that PEV is mediated by associations between homologous sequences (DORER and HENIKOFF 1994; SABL and HENIKOFF 1996). Local sequence repetitiveness is proposed to be sufficient to nucleate the condensed state of heterochromatin. This model was supported by the observation that heterochromatic variegation of a *P* transposon carrying a mini-*white* gene can occur as a consequence of local duplications of the transposon (DORER and HENIKOFF 1994). Silencing of *white*$^+$ (w^+) was strengthened with increases in the transposon copy number and with reversals in orientation of the transposon within the repeat array. Variegation was observed for an insertion site far removed from pericentric heterochromatin, demonstrating that a specific initiation sequence within the repeat array was not required for variegation. We proposed

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that gene silencing was caused by mislocalization of the transgene array to a heterochromatic compartment within the nucleus.

The possibility that compartmentalization is responsible for heterochromatic silencing of repeat arrays is consistent with the detection of long-range interactions between large blocks of heterochromatin along a chromosome (WAKIMOTO and HEARN 1990; EBERL *et al.* 1993; HENIKOFF 1997). The *brown*^{Dominant} (*bw*^{D}) allele, a large block of simple sequence DNA inserted into the coding region of the *brown* eye pigment gene, causes variegated silencing of the homologous copy of *brown*$^{+}$ (*trans*-inactivation, HENIKOFF and DREESEN 1989; HENIKOFF *et al.* 1995). *bw*^{D} is sensitive to a "heterochromatin distance effect" in that silencing is strengthened by X-ray-induced rearrangements that move the *bw*^{D} heterochromatic element closer to autosomal heterochromatin (TALBERT *et al.* 1994; HENIKOFF *et al.* 1995). Enhancement of *brown* variegation is correlated with increased frequency of association of the *bw*^{D} insertion with heterochromatin in both polytene (TALBERT *et al.* 1994) and diploid tissues (CSINK and HENIKOFF 1996; DERNBURG *et al.* 1996), and implies a causal relation.

If compartmentalization is important for PEV, then the size of the heterochromatic block inducing PEV might be expected to affect the degree of silencing. Surprisingly, this expectation was not fulfilled for the *white* gene. HOWE *et al.* (1995) used P transposase to generate deletions flanking a P[*w*$^{+}$] transposon next to an interstitial block of heterochromatin. These deletions had the same euchromatic breakpoint but varied in the heterochromatic breakpoints and thus the size of the heterochromatic block remaining. No correlation was seen between the extent of *white* variegation and the size of the block. However, local sequence differences resulting from differences in heterochromatic breakpoints might have masked a size effect.

Silencing effects induced by naturally occurring heterochromatin are complicated by its heterogeneity and uncertain composition. In contrast, mini-*white* repeat arrays are well defined and cause silencing effects that display two properties characteristic of heterochromatin (DORER and HENIKOFF 1994). First, the arrays caused variegated expression of *white* that resemble the typical mosaic phenotype caused by juxtapositions of the euchromatic *white* gene to heterochromatin. Second, the response of the variegation phenotypes to genetic modifiers of PEV demonstrated that gene silencing was related to heterochromatin-induced PEV. The arrays lack sequences specific for heterochromatin, suggesting that heterochromatin formation requires only repetitive DNA. Sequence repetitiveness as the basis for heterochromatin formation explains the extreme sequence diversity of constitutive heterochromatin, which includes both satellite and middle-repetitive sequences.

Silencing by artificial repeat arrays was observed only for the mini-*white* gene repeated within the array. However, in classical PEV, heterochromatin silences a single-copy reporter gene from a distance, and this has raised doubts about the relevance of our observations to PEV induced by natural heterochromatin (WEILER and WAKIMOTO 1995). Here, we show that a transgene array can cause silencing not only of the *white* reporter genes within the array, but also of a vital gene near the array. In these experiments, the size of the array inducing the effect correlates with the frequency of gene silencing, the first demonstration that changes in size of a heterochromatic block correlate with changes in PEV of a euchromatic gene. We also show that repeat arrays behave like natural heterochromatin in other ways. Just as for *bw*^{D}, increased silencing of a transgene repeat array results from rearrangements that place the array more proximal to pericentric heterochromatin. Linkage alterations are effective in *trans*, similar to the increased gene silencing by "homologue dragging" that has been demonstrated for *bw*^{D} (HENIKOFF *et al.* 1995; CSINK and HENIKOFF 1996). Thus, arrays as small as three copies of the transgene are sensitive to long-range effects of interactions with heterochromatin in both *cis* and *trans*. Finally, just as the *bw*^{D} heterochromatic element causes pairing-dependent *trans*-inactivation of *bw*$^{+}$, we observe that the transgene array is capable of silencing a non-variegating *white* transgene on a homologue.

MATERIALS AND METHODS

Drosophila stocks and crosses: Fly stocks were maintained on standard corn meal-molasses medium at 18° or at room temperature. All crosses for screens were carried out at 25°. Chromosomes and mutations not described in the text are described in Flybase (http://flybase.bio.indiana.edu:82). The P-*lacW* transposon repeat array lines were previously described (DORER and HENIKOFF 1994). *Df(2R)CX1, b pr/SM1* was obtained from the Bloomington, IN stock center; *Df(2R)CX1* is a deletion of the region from 49C1-4 to 50C23-D2 that uncovers the site of the 50C P-*lacW* repeat arrays inserted at 50C10-14 (data not shown). Lines bearing P-*lacW* insertions in *mastermind* were *mam*^{49} (1(2)1E7), *mam*^{50} (1(2)10E1) and *mam*^{51} (1(2)B11), and were kindly provided by H. RUOHOLA-BAKER.

To eliminate the P-*lacW* transposon repeat array from rearranged derivatives of 50C insertion chromosomes, *Sp/CyO; ry*^{506} *Sb P*[*ry*$^{+}$ Δ*2,3*] *99B/TM6, Ubx* males were crossed to *w*^{1118}; *P-lacW*[*50C*]*/CyO* females. *w*^{1118}*/Y; P-lacW*[*50C*]*/CyO; ry*^{506} *Sb P*[*ry*$^{+}$ Δ*2,3*] *99B/+* progeny were mated to *w*^{1118} females, and the resulting *Cy*$^{+}$ progeny were then examined for either white eyes (indicating loss of all P-*lacW* repeats) or pale orange eyes (indicating a reduction of the array to a single P-*lacW* transposon). To test for suppression of P[*brown*$^{+}$], *T(2;3)V21*^{t}-bearing flies with suppressed variegation of a P-*lacW* array at 92E were crossed to homozygous *w*$^{+}$; *bw*^{D} flies: in a *bw*^{D}*/bw*$^{+}$ background, suppression of P[*brown*$^{+}$] variegation is easily observed. To observe effects of the 50C P-*lacW* repeat arrays on viability, *w*^{1118}*/Y; P-lacW*[*50C*]*/CyO, pr* males were crossed to *Df(2R)CX1, b pr/SM1* females. In each bottle, 12 females were allowed to lay eggs for 6 days before being transferred to fresh bottles. Progeny were scored until 18 days after starting each bottle.

X-ray mutagenesis: Males were aged 3–5 days and exposed

to a single 3000-r dose of X-rays and crossed to virgin w^{1118} females. Four males were mated with 16–20 females per bottle. Progeny were raised in uncrowded bottles and examined for suppression of *white* variegation in the four-copy 92E P-*lacW* insertion line (nine bottles) and for enhancement of *white* variegation in three- and seven-copy 50C P-*lacW* insertion lines (16 bottles and 31 bottles, respectively), then mated to w^{1118} flies to determine heritability and w^{1118}; *CyO/Sp* flies to establish stocks.

Cytology: Polytene chromosomes in lines showing suppression or enhancement linked to P-*lacW* insertions were examined for rearrangements. Derivatives of the 92E four-copy P-*lacW* insertion line were balanced with *TM6B*, which bears the dominant larval marker *Tubby* (*Tb*). *Tubby*$^+$ larvae were selected for analysis after outcrossing rearrangement-bearing stocks to w^{1118}. Similarly, the dominant second chromosome larval marker *Black cell* (*Bc*) was used by outcrossing 50C P-*lacW* insertion lines to w^{1118}; *Bc Elp/CyO*. Males of the genotype w^{1118}/*Y*; *P-lacW*[*50C*]]/*Bc Elp* were then mated to w^{1118} females, and *Bc*$^+$ larvae were selected for analysis. Salivary gland squashes were performed as described (TALBERT *et al.* 1994) after raising larvae at 18° on instant food (Carolina Biological Supply) supplemented with yeast.

RESULTS

Suppressed variegation of a proximal mini-*white* transgene array: Tandem arrays of P-*lacW*, an enhancer-trap P transposon carrying the mini-*white* gene, cause variegated silencing of *white* that appears identical to heterochromatic PEV. Variegation of *white* has been described for a series of arrays located at cytological region 92E near the heterochromatic breakpoint of the rearrangement chromosome *T(2;3)V21*^{e} (DORER and HENIKOFF 1994). This chromosome has a euchromatic breakpoint at 92B on the right arm of chromosome *3* and a second break in the pericentric heterochromatin of the right arm of chromosome *2* (*2*R). A tandem array of four copies of P-*lacW* at 92E results in a strongly variegated eye phenotype, with red spots and clones on a white to pale orange background (Figure 1A).

Although a single copy of P-*lacW* at this position shows no variegation, the proximity of the transgene to a heterochromatic breakpoint raises the question of the importance of the nearby heterochromatin to the variegation phenotype of the four-copy array. Attempts to recombine the 92E array onto a normal sequence chromosome were not successful (data not shown). Instead, we irradiated a line with a four-copy P-*lacW* array and examined progeny of mutagenized flies for suppression of *white* variegation (Figure 2). Out of 1832 *T(2;3)V21*^{e}-bearing progeny, nine (0.5%) showed heritable suppression, five of these to an almost completely red eye. Four of the strongly suppressed lines were examined cytologically. Two of these lines have complex rearrangements from multiple new breaks: for these, cytological analysis was complicated by the difficulty in interpreting additional rearrangements superimposed on the homozygous lethal *T(2;3)V21*^{e}. In each of the other two lines, two new chromosomal breaks move the transgene array to a distal position on a different chromosome arm. In at least one line, heterochromatin is present at the new breakpoint, indicating that one break occurred in the *2*R heterochromatin proximal to the transgene array on *T(2;3)V21*^{e}.

Because *T(2;3)V21*^{e} also carries a strongly variegating P[*brown*$^+$] duplication at 92C, the lines with suppressed *white* variegation were also examined for *brown* expression (SABL and HENIKOFF 1996). In three of the four lines, *brown* variegation was suppressed, suggesting that the same linkage alterations may simultaneously affect variegation of the two transgene arrays. These results demonstrate that it is possible to profoundly alter silencing of genes within repeat arrays by changing the overall chromosomal linkage, even without changing the local sequence context. Evidently, the initial detection of variegation in this system was facilitated by the proximity of the array to pericentric heterochromatin.

Enhanced variegation of a medial mini-*white* transgene array: If proximity of the transgene array to heterochromatin is an important factor contributing to the degree of variegation, it should also be possible to select for enhancement of variegation of a more distal insertion. Suitable lines for this experiment were provided by a series of transposon arrays inserted at cytological position 50C10-14, a medial position on the right arm of a non-rearranged chromosome *2*. Variegation of *white* is observed for repeats of three or more P-*lacW* transposons at this site (DORER and HENIKOFF 1994). Two lines were subjected to X-ray mutagenesis, one with a seven-copy P-*lacW* repeat array and one with a three-copy insertion (Figure 2). For both of these, progeny with enhanced variegation of *white* were selected, and lines were established from those in which the enhancement of variegation was a consistent characteristic that segregated with the repeat array in outcrosses (Table 1 and Figure 1B). All lines subjected to polytene chromosome examination were found to contain chromosomal rearrangements. Some of the rearrangements are complex, involving multiple breaks. Lines that appeared to have only two breaks are summarized in Figure 3. In all 13 of these lines, one break is in the euchromatin of *2*R, and the second break is in either autosomal pericentric heterochromatin or in the heterochromatic *Y* chromosome.

The perfect correlation between enhancement and relocation of 50C repeat arrays closer to blocks of heterochromatin confirms that position along the chromosome arm is important for silencing, independent of the local sequence context. Either pericentric or *Y*-heterochromatin is effective. The recovery of three translocations moving *Y*-heterochromatin distal to 50C without changing the distance to the centromere demonstrates that enhancement does not depend on closer linkage to the centromere.

Modification caused by linkage changes: The heterochromatin distance effects described above for mini-*white* repeat arrays at 92B and 50C are similar to those

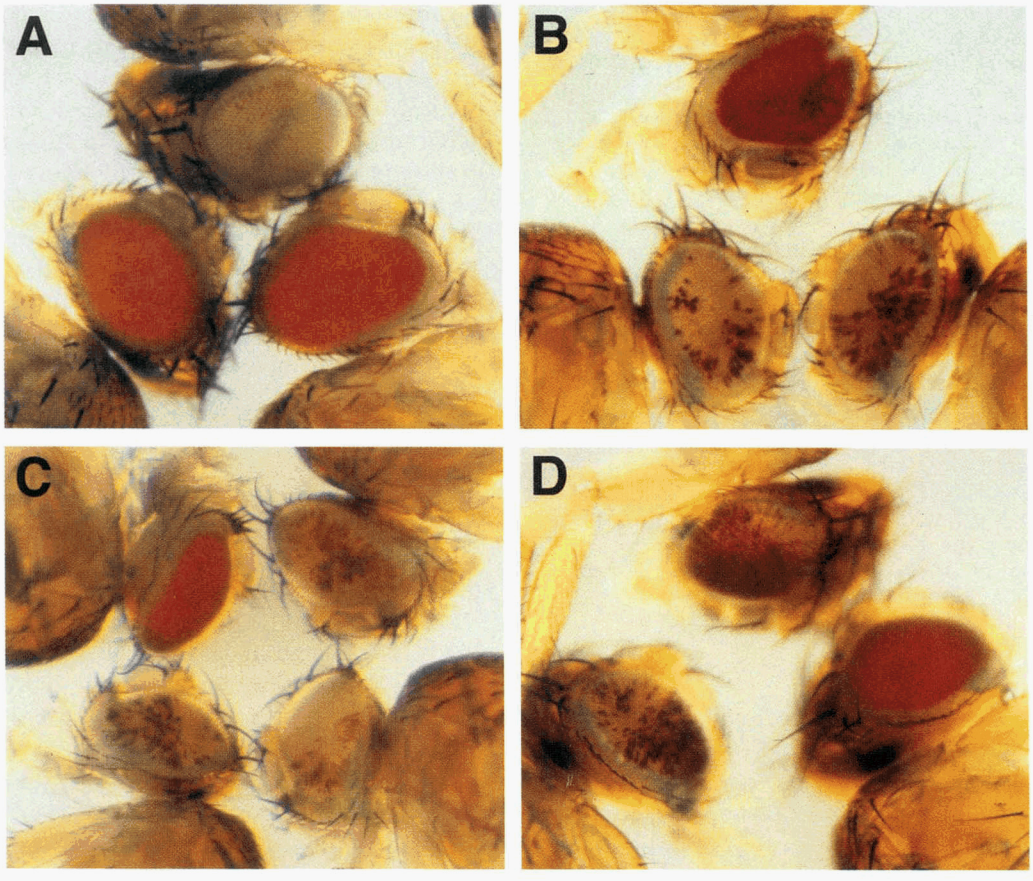

FIGURE 1.—Phenotypes of flies carrying mini-*white* repeat arrays. (A) Linkage suppression of 92E four-copy insertion. Top, original four-copy line. Lower left and right, two independent suppressed derivatives. (B) Linkage enhancement of 50C seven-copy insertion. Top, original seven-copy line. Lower left and right, two independent enhanced derivatives. (C) Heterochromatin distance effect is not caused by disruption of transvection. Upper left, original seven-copy 50C insertion line. Upper right, phenotypic enhancement by linkage modification in *cis*. Lower left, phenotypic enhancement by linkage modification in *trans*. Lower right, structural homozygote for rearrangement. (D) *trans*-inactivation by 50C repeat array. Lower left, heterozygote for six-copy array with single inverted transposon within the array. Lower right, heterozygote for tandem duplication. Top, heterozygous combination of the six- and two-copy insertions.

reported for rearrangements of bw^D (HENIKOFF *et al.* 1995). In that study, various criteria were applied to demonstrate that the linkage alterations were responsible for the phenotypic enhancement of *brown* variegation and to argue for the heterochromatin distance effect as the most likely explanation. We have applied the same criteria to the analysis of the transgene array. First, to test the possibility that enhancement of variegation is caused by a change in the transposon array, we separated the array from the rearrangement. This was carried out with four of the two-break P-*lacW* repeat lines (Table 2), which are normally maintained as balanced stocks. The lines were outcrossed, then females heterozygous for the rearranged insertion chromosome and a normal sequence chromosome were again outcrossed. An apparent "revertant" to the original, non-enhanced 50C insertion phenotype was selected in each line. In all four cases, the revertants had the normal second chromosome sequence. This indicates that enhancement of variegation was not caused by alteration of the transgene arrays during the X-ray mutagenesis and confirms the correlation between enhancement and chromosomal rearrangement.

Second, we tested whether the rearrangements act as general enhancers of PEV in *trans*. This could occur if the rearrangement breakpoints themselves caused mutations in PEV modifier genes (DORN *et al.* 1993), or if another change had occurred coincidentally. To test this possibility, we examined the effects of several rearrangements of P-*lacW* repeat lines on other *white* variegating alleles. Two different tests were carried out. In the first test, four different rearrangement chromosomes from lines showing the strongest enhancement of variegation were examined (Table 2). These chromosomes have different heterochromatic and euchromatic rearrangement breakpoints that would be expected to affect independent *trans*-acting enhancers of variegation. They were tested for possible interactions with two different *white*-variegating alleles that were chosen for consistently displaying significantly darker pigmentation than the strongly enhanced 50C insertion lines. One of the darker lines chosen was $In(1)w^{mottled4}$ (w^{m4}),

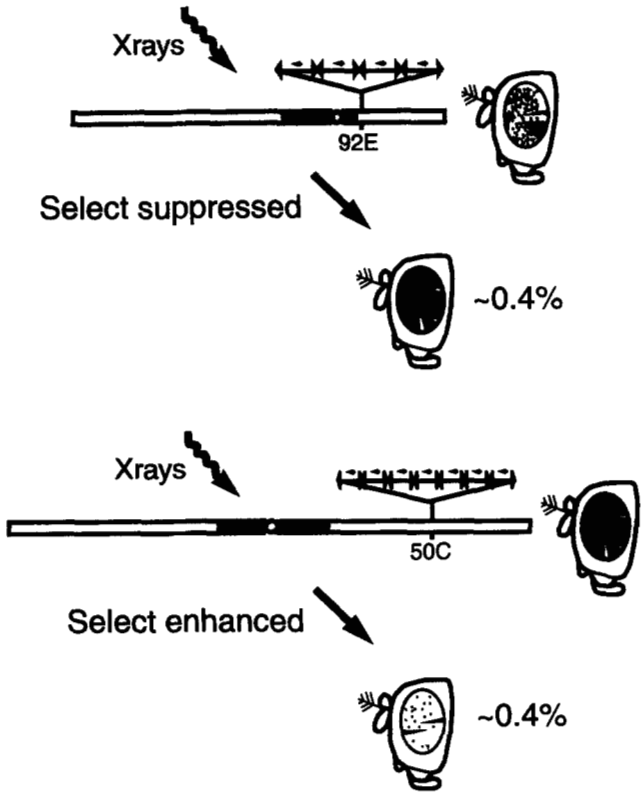

FIGURE 2.—Screens for suppression and enhancement of mini-*white* variegation. Above, a line carrying a four-copy P-*lacW* repeat array at a proximal position on a rearrangement chromosome was irradiated as described in the text, and progeny were examined for suppression of *white* variegation. Below, lines with three- and seven-copy arrays of P-*lacW* at a medial position on a normal sequence chromosome were irradiated, and progeny examined for enhancement of *white* variegation. □, euchromatin; ■, heterochromatin; ○, centromere. Transposon arrays are not shown to scale; their orientations relative to the chromosome are unknown.

which is widely used in genetic screens and in tests for modifiers of PEV (GRIGLIATTI 1991). The other was a *T(2; 3)V21*^{c} line carrying a three-copy P-*lacW* tandem array inserted at 92E, which produces a dark-mottled phenotype sensitive to PEV modifiers (DORER and HENIKOFF 1994). None of the four 50C insertion chromosomes caused any enhancement of variegation of *white*

TABLE 1

Screens for enhancement of *white* variegation

	P-*lacW* copy number	
	3	7
Progeny screened	7584	5875
Total enhanced	21 (0.28)	52 (0.89)
Sterile	9	28
Linked enhancers	12 (0.16)	24 (0.41)
X-linked	0	2
Y-linked	1	5
Autosomal	11	17

Values in parentheses are percentages.

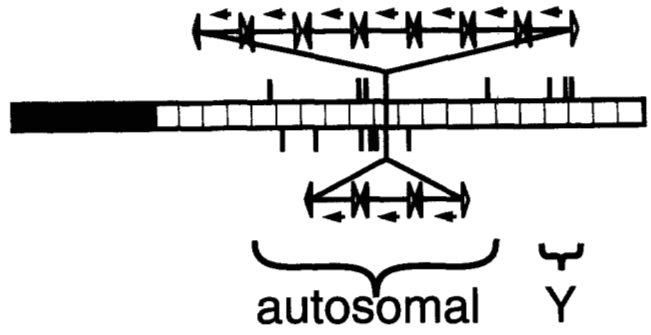

FIGURE 3.—Distribution of chromosomal breakpoints in rearrangements causing enhancement of variegation. The position of 50C transposon arrays is shown in 2R euchromatin (□), which is demarcated to indicate Bridges' Divisions 41–60. ■ represents the pericentric heterochromatin of 2R. The lines above and below the chromosome arm indicate the euchromatic breakpoint positions of two-break enhancing rearrangements involving seven- and three-copy arrays, respectively, which are depicted schematically at 50C. All rearrangements included within the larger bracket had a second breakpoint within autosomal heterochromatin, while the remaining three had a second break within the heterochromatic *Y* chromosome.

in either the w^{m4} inversion or the 92E transgene array. In the second test, to remove the possibility of complications caused by expression of *white* from the 50C transgene array, P transposase was used to eliminate the transgene array from three of the translocation chromosomes. These chromosomes failed to enhance the variegation of the three-copy 92E P-*lacW* insertion. These experiments demonstrate that enhancement is specific for the transgene array at 50C and does not result from mutations in modifiers of PEV.

Third, we asked whether linkage alterations enhance variegation because of disruption of pairing between homologous chromosome arms. This would be similar to disruption of transvection or pairing-dependent complementation (LEWIS 1954). Transvection is disrupted by breaks between the centromere and the reporter gene for either homologue in a heterozygote and is restored when those rearrangements are homozygous (LEWIS 1954; GELBART 1982; LEISERSON *et al.* 1994). That enhancement of 50C variegation is not due to transvection is suggested by the recovery of both proximal and distal breaks, even though the latter would not disrupt transvection. We have tested the transvection hypothesis more directly by making flies homozygous for enhancing rearrangements. As noted above, P transposase was used to remove the transgene array from three rearrangement lines. For two of the rearrangements, the breakpoints were homozygous viable, and it was possible to combine one chromosome without the transgene array with a second that carried the array. Restoration of transvection would have resulted in the original variegating phenotype. However, the effect on *white* variegation was usually in the opposite direction; that is, enhancement was as strong or

TABLE 2

Linkage changes causing enhancement of *white* variegation in P-*lacW* arrays at 50C

Array size	Breakpoints	Linkage reversion?[a]	General modifier of PEV?	Enhancement of 50C P-*lacW* variegation in *trans*?[b]
7	49BC; 3het	Yes	No[c]	ND
7	49D; 3het	Yes	No[d]	Yes
3	50A; 2Lhet	Yes	No[c]	ND
7	54D; 2Rhet	Yes	No[d]	Yes[e]
7	42A; 51F; 2het	ND	No[c,d]	Yes[e]
7	*T(2;3)*[f]	ND	No[c]	ND
7	45EF; 2Lhet			
3	46B; het			
3	47D; 3Rhet			
3	49C; het			
3	49F; 2het			
3	51C; 2Rhet			
7	57AB; *Y*			
7	57F; *Y*			
7	58A; *Y*			
7	*T(2;3)*[f]			
7	*T(Y;2;3)*[f]			
7	*T(Y;2;3)*[f]			

All lines subjected to polytene chromosome analysis are listed. Further tests were performed on six lines, except where not done (ND) as noted.

[a] Recombination of 50C P-*lacW* array from rearrangement onto a normal-sequence chromosome is accompanied by reversion of phenotype.

[b] Rearrangement chromosomes with all copies of P-*lacW* removed from 50C were tested with a seven-copy P-*lacW* insertion at 50C.

[c] Rearrangement chromosomes carrying a seven-copy P-*lacW* array at 50C were tested for enhancement of variegation of w^{m4} and of a three-copy P-*lacW* insertion at 92E.

[d] Rearrangement chromosomes with all copies of P-*lacW* removed from 50C were tested for enhancement of variegation of a three-copy P-*lacW* insertion at 92E.

[e] Homozygotes are viable and show enhancement of variegation.

[f] More than two breaks, breakpoints not determined.

stronger than when the rearrangement was paired with a normal sequence chromosome. These observations are similar to what has been seen for bw^D, where approximate restoration of pairing typically results in silencing as strong or stronger than for unpaired heterozygotes (HENIKOFF *et al.* 1995).

We also asked whether the array and the rearrangement must be on the same chromosome, or can be on the homologous chromosome. In the case of bw^D it was shown that enhancement occurred even when the rearrangement was in *trans* to the heterochromatic insertion, suggesting "homologue dragging" of bw^D into contact with proximal heterochromatin. Indeed, the heterozygous combination of the *white*$^-$ rearrangement chromosome with the original, non-rearranged seven-copy chromosome produced enhancement of P-*lacW* variegation in all three tested cases (Table 2; Figure 1C). Thus, increased proximity of the transgene array to heterochromatin in either *cis* or *trans* can increase the strength of transgene silencing.

Heterochromatic silencing of a nearby essential gene: The non-rearranged chromosomes carrying P-*lacW* insertions at 50C are each homozygous viable. As described above, it is possible to make structural homozygotes for two of the rearrangements that enhance the P-*lacW* variegation. However, in neither of these cases was it possible to create flies homozygous for both the rearrangement and the transgene array: the structural homozygotes only survived when the array was removed from one homologue using P transposase. This suggested that a nearby essential gene or genes is affected by the transgene insertion. If there is variegation of an essential gene near the array, then genetic alterations that enhance *white* variegation will also enhance lethality. To test this, chromosomes with different-sized arrays were made heterozygous with a chromosome bearing a deficiency for the 50C region, *Df(2R)CX1*. Whereas lower copy number insertions survived well in combination with the deficiency, the heterozygotes containing higher copy number insertions were recovered at significantly reduced frequencies (Table 3). Thus, an increase in transposon copy number is associated with both an enhancement of *white* variegation and an increase in the lethal effect.

It is possible that disruption of gene function by the insertion arrays, rather than heterochromatin-mediated silencing, is responsible for the increase in lethality with increasing array size. To test this, we compared the

TABLE 3

Viability of P-*lacW* insertion lines

Array size	Breakpoints	P-*lacW[50C]*/Df(2R)CX1*	*CyO/Df(2R)CX1*	Viability[a]
2	None	270	278	0.97
3	None	311	365	0.85
4	None	324	533	0.61
7	None	20	599	0.03
3	51C; 2Rhet	6	331	0.02
1	51C; 2Rhet	72	78	0.92
3	47D; 3Rhet	23	245	0.09
1	47D; 3Rhet	59	100	0.59

[a] Ratio of *P-lacW[50C]*/Df(2R)CX1, b pr* (Cy^+, pr^+) to *CyO, pr/Df(2R)CX1, b pr* (Cy, pr) siblings.

viability of two linkage-enhanced three-copy lines to the parental three-copy line, which is almost fully viable. In both cases, viability was dramatically decreased by the presence of the rearrangement that enhances the variegation of the *white* reporter gene (Table 3). Both rearrangements have breakpoints outside the region deleted by *Df(2R)CX1,* so the lethality is not expected to result from new lesions within that region. This was confirmed by reducing the array to a single copy of P-*lacW* on both chromosomes, which greatly increased their viability (Table 3). In all cases, increased lethality was correlated with enhanced variegation, whether caused by expansion of the transgene array or by alterations in chromosome linkage. We conclude that an essential gene or genes in the 50C region is silenced by the presence of a variegating mini-*white* array.

Transgene arrays *trans*-inactivate: Some heterochromatic insertions, such as bw^D, cause silencing of a wild-type gene present on a paired homologue, referred to as "*trans*-inactivation" (HENIKOFF and DREESEN 1989). We suspected that variegating mini-*white* arrays at 50C also cause *trans*-inactivation, based on examination of homozygotes. Homozygotes carrying a two-copy P-*lacW* insertion have non-variegated eyes with visibly more pigment than is seen in eyes of two-copy hemizygotes. In contrast, homozygotes for variegating six- and seven-copy insertions show less pigment than the corresponding hemizygotes. To determine whether or not this unexpected behavior of homozygotes is an example of *trans*-inactivation, we examined heterozygous combinations of different-sized arrays. Six- and seven-copy arrays were dominant to the two-copy insertion, with heterozygotes showing variegated eyes (Figure 1D). The eyes of heterozygotes for single-copy insertions are too pale to allow an unambiguous determination if they are also *trans*-inactivated by variegating arrays on normal sequence chromosomes. However, the heterozygous combination of a single-copy allele and a seven-copy array strongly enhanced by linkage alterations is phenotypically paler over most of the eye than the single-copy allele alone (not shown). Because we detected no variegation in single-copy homozygotes subject to linkage enhancement, we conclude that the array is required for *trans*-inactivation of a single-copy allele.

As described above, there is no effect of the rearrangement lines derived from the 50C insertions on a three-copy P-*lacW* array at 92E, suggesting that *trans*-inactivation by the 50C arrays is specific for *white* at the homologous insertion site. This was further tested with three other P-*lacW* transposons independently inserted in the *mastermind* (*mam*) locus at 50D1, a position somewhat distal to the variegating array inserted at 50C10-14. These transgene insertions also failed to be *trans*-inactivated by two different variegating 50C arrays, consistent with a strict requirement for homologous positioning in *trans*-inactivation (HENIKOFF *et al.* 1993).

DISCUSSION

Heterochromatin and transgene repeat arrays have similar properties: Heterochromatin displays a number of properties that reflect the ability to interact with euchromatic genes and with other heterochromatic sequences, even at large distances. Both kinds of interactions can be observed in both *cis* and *trans*. The inactivation of euchromatic genes provides a measure of the strength of these interactions. Our previous experiments showed that a Drosophila transgene that did not contain typical heterochromatic satellite DNA or other naturally repetitive sequences acquired properties of heterochromatin when tandemly repeated (DORER and HENIKOFF 1994). The repeated transgenes were silenced in a variegated pattern that resembled classical PEV and were sensitive to known modifiers of PEV. However, the relevance of these observations to heterochromatin-induced PEV was uncertain (WEILER and WAKIMOTO 1995), because classical PEV causes silencing of single-copy genes near heterochromatin, whereas transgene silencing that we reported was restricted to a multi-copy reporter within the array. Furthermore, differences between sites of transgene arrays might have resulted from relative proximities to heterochromatin or might instead have been caused by differences in local sequence context. To address these questions, we have asked whether transgene repeat arrays possess ad-

ditional properties of heterochromatin. Indeed, we find that transgene repeat arrays act as interstitial blocks of heterochromatin, interacting with euchromatic genes and natural heterochromatic sequences in both *cis* and *trans*.

Size of the heterochromatic block correlates with degree of silencing: The decrease in viability of flies heterozygous for large, but not small, transgene arrays at 50C and a deletion of the chromosomal region shows that heterochromatic silencing includes at least one vital endogenous gene. Like the silencing of *white* transgenes within the array, this spreading of inactivation correlates not only with proximity to heterochromatin, but also with the size of the array. HOWE *et al.* (1995) have shown previously that effects on euchromatic gene silencing vary with different breakpoints in the flanking heterochromatic block without being correlated with the size of the block. Because of sequence heterogeneity of heterochromatin in naturally occurring blocks, it was not feasible to make changes in size alone. However, in the present experiments with artificial heterochromatic arrays, we used P transposase to vary the overall size of the repetitive array without changing the sequence composition. Our results demonstrate a size correlation. In the experiments of HOWE *et al.* (1995), effects due to differences in sequence composition of the block may have masked any effect resulting from changing its size.

Transgene arrays respond to heterochromatin distance effects: In previous work, a difference was seen in the extent of *white* gene silencing between comparable transgene arrays at a proximal site and a medial site (DORER and HENIKOFF 1994). Here, we have demonstrated that such differences depend on proximity to blocks of heterochromatin and are examples of heterochromatin distance effects. Expression of *white* from arrays at either insertion site varies over a wide range of pigment levels depending on linkage, even though the local sequence context for each insert remains unchanged. Furthermore, the sensitivity of these phenotypes to heterochromatin distance effects supports the hypothesis that the transgene repeats are being silenced by the formation of artificial heterochromatin.

Silencing of *brown*$^+$ by *bw*^{D} was shown to be enhanced by linkage alterations in *trans* as well as in *cis* (HENIKOFF *et al.* 1995). This is explained by a homologue-dragging model, which proposes that somatic pairing between homologues pulls the *bw*^{D} heterochromatic element on the normal-sequence chromosome to a more proximal position, causing it to be susceptible to a heterochromatin distance effect on gene silencing (HENIKOFF *et al.* 1995). The effects of homologue dragging on the nuclear localization of *bw*^{D} can be observed cytologically in diploid larval nuclei (CSINK and HENIKOFF 1996). Silencing of transgene arrays at 50C is also sensitive to linkage modifications in *trans*, suggesting that the same model may be generally applied to natural interstitial heterochromatin and to artificial heterochromatin at transgene insertions.

Heterochromatin distance effects suggest general stickiness of heterochromatin: DERNBURG *et al.* (1996) and CSINK and HENIKOFF (1996) measured the association of sequences near *brown* with a block of AACAC satellite sequence located in proximal 2R, the same chromosome arm containing the distal *bw*^{D} element. Because the *bw*^{D} heterochromatic insertion is composed primarily of AAGAG repeats, and the proximal heterochromatin of chromosome *2* contains the bulk of the AAGAG satellite sequence in the fly genome (LOHE *et al.* 1993), DERNBURG *et al.* (1996) proposed that the increased association of the distal 2R sequences to proximal chromosome *2* in *bw*^{D} chromosomes may be mediated by sequence-specific interactions between the different large stretches of AAGAG. Alternatively, longer-range interactions may result from general cohesion of heterochromatin such that it has a tendency to associate with other heterochromatin with little regard to specific sequence. Such "stickiness" more easily accounts for the enhancement of 50C *white* variegation, as the P-*lacW* transposon does not contain repeats of natural satellite DNA or other sequences present in pericentric heterochromatin. Furthermore, rearrangements that enhance either *bw*^{D} or *white* variegation can involve any autosomal arm, suggesting that linkage to a particular heterochromatic sequence is not required. Also, *white* variegation associated with the transgene arrays at 50C is enhanced in *T(Y;2)* rearrangements with distal 2R breaks, indicating that the transgene array is sensitive to heterochromatin of the *Y* chromosome. The recovery of distal 2R breaks that move *Y* heterochromatin closer to transgene arrays also reveals that heterochromatin distance effects result from proximity to heterochromatin and not proximity to the centromere as proposed by DERNBURG *et al.* (1996).

The demonstration of heterochromatin distance effects for P-*lacW* arrays with as few as three copies of a ~10-kb transposon extends the generality of observations made using *bw*^{D}, a megabase-sized heterochromatic insertion composed largely of tandem pentameric repeats. Although we have no cytological evidence as yet of associations between P-*lacW* arrays and heterochromatin, the striking similarity between P-*lacW* and *bw*^{D} in showing heterochromatin distance effects suggests that associations similar to what have been observed for *bw*^{D} also occur for P-*lacW*.

Trans-inactivation by artifical heterochromatin at transgene arrays: Transgene arrays at 50C show the ability to *trans*-inactivate mini-*white* transposons at the homologous insertion site. As with the silencing of a vital endogenous gene in *cis*, these effects increase as variegation of the transgene arrays is enhanced. The ability to cause *trans*-inactivation is further evidence that mini-*white* transgene arrays form heterochromatin akin to natural heterochromatin.

Susceptibility to *trans*-inactivation has been observed for only a few Drosophila genes, and only *brown* has been extensively studied (HENIKOFF *et al.* 1993). Recent evidence shows, however, that *white* transgenes also can be susceptible to silencing by heterochromatin in *trans* (MARTIN-MORRIS *et al.* 1997). *Trans*-inactivation of *brown* depends on homologous pairing between the rearranged, inactivating chromosome and its homologue bearing the *brown* target gene. The inability of the 50C transgene arrays to silence the *white* reporter in P-*lacW* insertions at 50D and 92E suggests that pairing is also required for *trans*-inactivation by heterochromatic transgene repeats.

Repeat-induced gene silencing and heterochromatin formation: Repeat-induced gene silencing (RIGS, ASSAAD *et al.* 1993) has been observed in other organisms, including fungi and plants (FLAVELL 1994; ROSSIGNOL and FAUGERON 1995). In vertebrates, transgene insertions frequently produce mosaic and low-expressing phenotypes that appear to vary between insertions of the same transgene (reviewed in MARTIN and WHITELAW 1996; DORER 1997). These difficulties in obtaining consistent expression may be attributed to chromosomal position effects; however, these effects are not always easy to evaluate because of rearrangements that frequently occur in the insertion of vertebrate transgenes. One common rearrangement is the insertion of a large repeat array of a transgene at a single site. By analogy with our demonstration of RIGS in Drosophila (DORER and HENIKOFF 1994), these other silencing phenomena could be interpreted as heterochromatic effects. The extent to which a transgene array is silenced may then depend in an inverse way on copy number as well as on its integration site (DOBIE *et al.* 1996). The interpretation that silencing results from the formation of heterochromatin at repeat arrays is further supported by the detection of an altered chromatin configuration associated with RIGS in Arabidopsis (YE and SIGNER 1996). The ability of some transgenes to be consistently expressed in a copy-number-dependent manner independent of insertion site may require locus control region (LCR) sequences for the maintenance of an open chromatin structure and the inhibition of heterochromatic silencing (FESTENSTEIN *et al.* 1996; MILOT *et al.* 1996). If heterochromatin is generally responsible for RIGS in eukaryotes, we anticipate that the effects reported here, including spreading, long-range associations with natural heterochromatic sequences and *trans*-inactivation, will be detected in transgenic eukaryotic organisms in addition to Drosophila.

In Drosophila, where powerful cytological and genetic tools are available, studies of heterochromatin and PEV have been hampered by the enormous size and heterogeneity of heterochromatic blocks and the difficulty in molecular analysis of repetitive DNA. Our demonstration that mini-*white* repeat arrays behave identically to natural heterochromatin provides an experimentally attractive system for studying heterochromatin and PEV. The heterochromatic blocks are small, molecularly defined and can be easily expanded and contracted at a site, with phenotypic selection for screening. These advantages of the system have allowed us to detect the effect of size of a heterochromatic block on the strength of gene silencing. We expect that further studies using transgene repeat arrays will allow for a more precise description of heterochromatin at the molecular level.

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