A Reexamination of Spreading of Position-Effect Variegation in the *white-roughest* Region of *Drosophila melanogaster*

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ABSTRACT

In Drosophila, heterochromatin causes mosaic silencing of euchromatic genes brought next to it by chromosomal rearrangements. Silencing has been observed to "spread": genes closer to the heterochromatic rearrangement breakpoint are silenced more frequently than genes farther away. We have examined silencing of the *white* and *roughest* genes in the variegating rearrangements $In(1)w^{m4}$, $In(1)w^{mMc}$, and $In(1)w^{m51b}$. Eleven stocks bearing these chromosomes differ widely in the strength of silencing of *white* and *roughest*. Stock-specific differences in the relative frequencies of inactivation of *white* and *roughest* were found that map to the *white-roughest* region or the adjacent heterochromatin. Most stock-specific differences did not correlate with gross differences in the heterochromatic content of the rearranged chromosomes; however, two stocks, $In(1)w^{m51b}$ and $In(1)w^{mMc}$, were found to have anomalous additional heterochromatin that may act *in trans* to suppress variegating alleles. In comparing different stocks, the frequency of silencing of the *roughest* gene, which is more distant from heterochromatin, does not correlate with the frequency of silencing of the more proximal *white* gene on the same chromosome, in contradiction to the expectation of models of continuous linear propagation of silencing. We frequently observed rough eye tissue that is pigmented, as though an active *white* gene is skipped.

IN his pioneering study of X-ray mutagenesis in *Drosophila melanogaster*, H. J. Muller (1930) discovered a new class of mutations that could simultaneously affect the expression of multiple genes in the same chromosomal region. For example, the mutation w^{m1} resulted in the mosaic expression both of the *white* (*w*) gene, which controls the formation of the red eye pigments, and of the nearby *Notch* (*N*) gene, which is necessary for differentiation of ectodermal derivatives. This type of variable gene inactivation is known as position-effect variegation (PEV), which typically occurs when euchromatic genes are brought next to pericentric heterochromatin by chromosome rearrangements (Schultz 1936).

Schultz (1939) offered the first interpretation of how such variegating rearrangements can affect multiple linked genes at a distance from the rearrangement breakpoints. He noted the importance of studying the expression of two neighboring genes (*w* and *N*) in the same tissue (the eye). Using the translocation $T(1;4)w^{m258\text{-}21}$, Schultz found that wherever the gene more distant from the breakpoint (*w*) showed a mutant phenotype, the gene closer to the breakpoint (*N*) did also, although the converse was not observed. This relationship was also noted in the work of Demerec and Slizynska (1937) on the translocation $T(1;4)w^{m258\text{-}18}$, which affects *w* and the nearby eye-patterning gene *roughest* (*rst*) in a similarly ordered manner. Schultz explained these results by hypothesizing an inactivation process that "spreads" from the heterochromatic breakpoint. He also observed a cytological correlate of this spreading effect in polytene chromosomes: the euchromatic bands adjacent to the $T(1;4)w^{m258\text{-}21}$ heterochromatic breakpoint became darkened, disarranged, and "in some cases completely heterochromatic" (Schultz 1939, p. 261).

A molecular model currently popular for explaining the genetic phenomenon of spreading was developed by Tartof and co-workers (Tartof *et al.* 1984, 1989; Locke *et al.* 1988), who have characterized their model as an "oozing model" (Ptashne 1986). In this model, heterochromatin-specific proteins bind to initiator sequences within the heterochromatic DNA and propagate continuously along the chromosome from the initiation sites, forming compacted and genetically inert chromatin. In wild-type chromosomes, these proteins are confined to the heterochromatic DNA sequences either by terminator sequences or by proximally directed initiators. In rearranged chromosomes that juxtapose euchromatic and heterochromatic regions, however, initiators may direct assembly of heterochromatin-specific proteins so that they propagate into adjacent euchromatic sequences in a directional manner. Variegation is explained by the different extent to which this propagation occurs in each cell.

Despite the appeal of the oozing model, recent observations appear to contradict its underlying assumptions.

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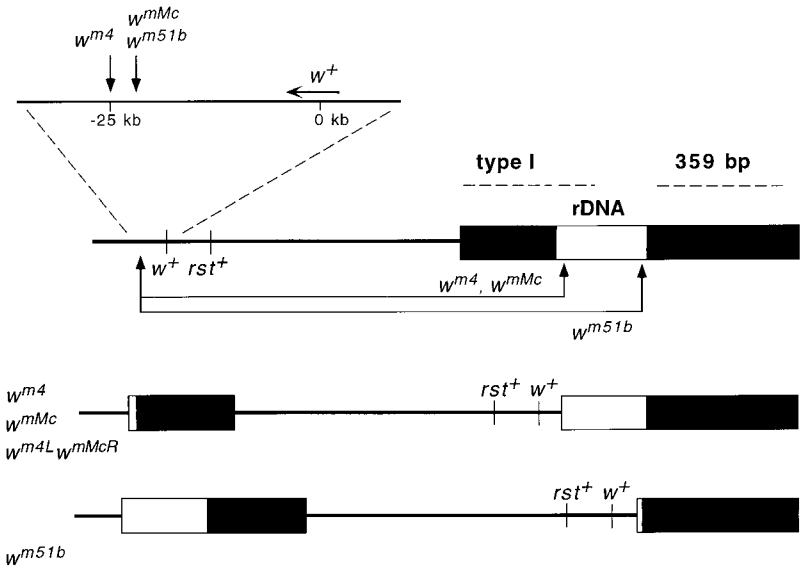

Figure 1.—Schematic diagrams of the w^{m4}, w^{mMc}, and w^{51b} inversions. The top line shows a magnified view of the region around the *w* gene. Other solid lines represent the *X* chromosome, which is not drawn to scale. Dashed lines indicate the location of type I and 359-bp repeats. Open boxes, ribosomal DNA repeats; filled boxes, other heterochromatin; vertical arrows, inversion breakpoints; horizontal arrow, the *w* transcription unit. Data from Appels and Hilliker (1982) and Tartof *et al.* (1984).

Since heterochromatic initiators in this model are imagined to be absent from euchromatic sequences, the propagation of the heterochromatin-binding proteins in the euchromatic regions must be continuous from the nearest heterochromatic initiator. In apparent contradiction to this requirement, Belyaeva and Zhimulev (1991) observed that compaction of polytene bands near heterochromatin can be discontinuous. In addition, the existence of the hypothesized heterochromatic initiators is called into question by the finding that repeated arrays of *P* transposons embedded in euchromatin may form heterochromatin at sites distant from pericentric heterochromatin, although the same *P* transposons do not initiate heterochromatin formation as single or double insertions (Dorer and Henikoff 1994). Finally, the assumption that strength of silencing depends upon distant determinants is questioned by the finding that purely local determinants are involved; for example, the strength of silencing is altered simply by reversing the orientation of a transgene reporter located ~60 kb away from a heterochromatic breakpoint (Sabl and Henikoff 1996). In view of these confounding observations, we chose to reexamine the spreading effect in the well-studied region of the *w* and *rst* genes using molecularly characterized rearrangements.

Tartof *et al.* (1984) investigated the structures of *In(1)*w^{m4}, *In(1)*w^{mMc}, and *In(1)*w^{m51b} (Figure 1). The euchromatic breakpoints of these three inversions (hereafter abbreviated as w^{m4}, w^{mMc}, and w^{m51b}) are found at coordinates −24.5 kb (w^{m4}) and −21 kb (w^{mMc} and w^{m51b}) ~25 kb downstream from the *w* transcription start site (+3.8). The heterochromatic breakpoint of w^{m51b} is in an unidentified repeated element in the proximal portion of the nucleolus organizer (NO), while the breakpoints of w^{m4} and w^{mMc} are in type I repeats located within the distal portion of the NO (Appels and Hilliker 1982; Tartof *et al.* 1984). The breakpoints of the latter two inversions are likely to be in similar positions: exchanging their left ends has no effect on their phenotypes (Muller 1946), even though a similar exchange between the left ends of w^{m4} and w^{m51b}, which changes the dosage of the NO, has measurable phenotypic effects (Spofford and DeSalle 1991). Given the structural similarities of these three inversions, we considered them to be good choices for investigating the factors that influence the spreading of gene inactivation from heterochromatin into adjacent euchromatin.

Using these inversions, we find that spreading occurs in a rearrangement-specific manner that is inconsistent with simple oozing models and appears to be discontinuous. We show that the genetic factor(s) that determine rearrangement-specific spreading is tightly linked to the euchromatin / heterochromatin junction, but that these factors do not correlate with the number or kinds of major repeats in the adjacent heterochromatin. We discuss the implications of these findings for models of PEV and the spreading process.

MATERIALS AND METHODS

Fly stocks: All flies were raised at 25° on standard cornmeal / molasses / agar medium. All mutations are described in Lindsley and Zimm (1992), except the revertant $w^{m4hx537}$ (Reuter *et al.* 1985) and the w^{m4} revertants generated in this study (see below).

We acquired stocks described as carrying w^{m4}, w^{mMc}, or the recombinant chromosomes $w^{mMcL}w^{m4R}$ and $w^{m4L}w^{mMcR}$ from various sources. The inversion breakpoints of these stocks (collectively referred to as w^m stocks) were determined by Southern analysis to confirm their identities. Several stocks proved to be labeled with inaccurate genotypes. Three chromosomes that putatively were recombinants between the left and right ends of w^{m4} and w^{mMc} retained both w^{m4} breakpoints (Figure 2, *4b*, *4f*, and *4r*). Two stocks were labeled *In(1)*w^{m4}, w^1, but both lacked the *Doc* insertion element and white-eyed phenotype

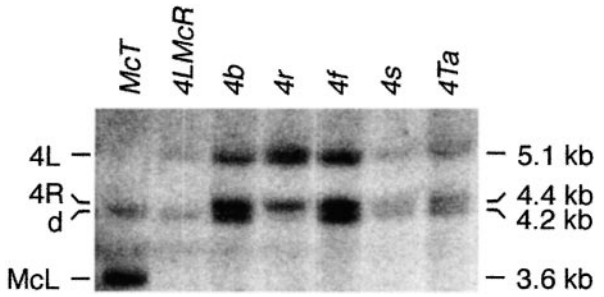

Figure 2.—Mapping of inversion breakpoints in putative recombinant chromosomes. Genomic DNA was digested with *Hin*dIII and probed with pWM4. 4L, the 5.1-kb junction fragment from the left end of the w^{m4} inversion; 4R, the 4.4-kb junction fragment from the right end of the w^{m4} inversion; McL, the 3.6-kb junction fragment from the left end of the w^{mMc} inversion; d, the 4.2-kb fragment from coordinates −31 to −27 kb, distal to the left end of the w^{m4} inversion. Stock *4r* has a polymorphism for this fragment.

that characterize the w^{1} allele (data not shown). For convenience and clarity, a unique designation reflecting these observations was assigned to each chromosome and the stock carrying it, as detailed in Table 1.

Stock *4e* was selected as a spontaneous extreme w^{m4} variant and has resided in our lab for more than a decade. Stock *4f* appears to have acquired a suppressing mutation during the two years that it has resided in our lab, based on a change both in its level of *w* inactivation and in its modification ranking (see below). Two closely related stocks, *4T* and *4Ta*, were derived from a w^{m4} / *Y Su(var)* stock (from K. Tartof) that has a moderate level of *w* inactivation. To replace the *Y Su(var)* chromosome, females from the latter stock were crossed to w^{mMc} / *Y* males from the *McT* stock (Table 1), and their w^{m4} / w^{mMc} daughters were backcrossed to w^{mMc} / *Y* males. The F_2 sibs were then crossed, and w^{m4} / w^{m4} females and w^{m4} / *Y* males from the F_3 generation (identified by moderate rather than weak levels of *w* inactivation) were mated in single pairs. One pair that bred true for a moderate level phenotype, in which unpigmented ommatidia occur both singly and in patches, was used to establish stock *4T*. Subsequently, a single male from stock *4T* was crossed to w^{1118} females and then to his w^{m4} / w^{1118} daughters. w^{m4} / *Y* males and w^{m4} / w^{m4} females from the F_2 generation were crossed to establish stock *4Ta*, derived from a single w^{m4} chromosome.

Phenotypic analyses: Eye phenotypes of *w*, *rst*, and *brown* (*bw*) mutations were assessed using standard dissecting microscopes. Modification of *In(2R)*bw^{VDe2} (hereafter bw^{VDe2}) was ranked by selecting five flies from the various w^{m}/+; bw^{VDe2} / + flies scored to act as standards representing the range of phenotypic variation among all such flies. The remainder of the w^{m} / +; bw^{VDe2} / + flies were classified as being most similar to one of the five standards by a technician who was otherwise unfamiliar with the w^{m} stocks involved.

Pigment assays for pteridines were performed on samples of one to five heads from flies aged 7–10 days. The heads were soaked for 3 days in 10 μl of 30% acidified ethanol before optical density measurements were made on a spectrophotometer (Beckman, Fullerton, CA) at 480 nm in a capillary cuvette. Two measurements were made from each sample, and 8–10 samples were measured for each genotype. The mean for all measurements of each genotype was calculated and normalized for the number of heads used. Correlation of pigment measurements with penetrance of nonrough eye tissue was analyzed using Statview 4.5 statistical software.

Recombination mapping: *4LMcR / Df(YS)bb* males were crossed to *4r* females, and the F_1 heterozygous *4r / 4LMcR* female progeny were allowed to mate with their *4r / Df(YS)bb* sibs. F_2 male progeny were collected and individually crossed first to *C(1)M4 / 0* females and then to *C(1)M4 / Y* females to establish lines. Of the F_2 males recovered, 36 were classified as having the right end of *4LMcR*, and 53 were classified as having the right end of *4r* based on the degree of *w* inactivation (moderate or weak, respectively) in their eyes. The inactivation level of *rst* in a sample of *X / 0* descendents of the F_2 males was determined by counting the number of eyes with and without patches of rough ommatidia. In some cases the sample of F_3 *X / 0* male progeny of a particular F_2 male was supplemented by crossing F_3 *X / Y* males from the corresponding established line to *C(1)M4 / 0* females to generate F_4 *X / 0* males.

TABLE 1

***white*-variegating stocks**

Stock	Inversion breakpoints	Original stock description	Source[a]
51b	*In(1)*$w^{m51bL}w^{m51bR}$	*In(1)*w^{m51b},w^{m51b}*ct*	L. G. Robbins
McB	*In(1)*$w^{mMcL}w^{mMcR}$	*In(1)*w^{mMc},w^{mMc} */ C(1)DX,yf*	BL 4478[b]
McT	*In(1)*$w^{mMcL}w^{mMcR}$	*In(1)*w^{mMc},w^{mMc}	K. Tartof
4LMcR	*In(1)*$w^{m4L}w^{mMcR}$	*In(1)*$w^{m4L}w^{mMcR}$,w^{mMc}*v / Df(YS)bb*	MA 1130[b]
4b	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*$w^{m4L}w^{mMcR}$,*y* w^{mMc} *vf*	MA 1131
4e	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*w^{m4},w^{m4} extreme variant	S. Henikoff[c]
4f	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*$w^{mMcL}w^{m4R}$,w^{m4}*f / Df(YS)bb*	MA 1121[b]
4h	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*$w^{m4h}w^{m4h}$*; EC66 / TM3*	G. Reuter
4r	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*$w^{m4L}w^{mMcR}$,*y* w^{mMc} *vf / Df(YS)bb*	E. Tolchkov
4s	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*w^{m4},w^{m4} */ Df(YS)bb Su(var)5*	MA 1126[b]
4T	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*w^{m4},w^{m4}	P. Talbert[c]
4Ta	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*w^{m4},w^{m4}	P. Talbert[c]
4w	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*w^{m4},$w^{m4}w^{1}$ */ Df(YS)bb*	MA 1123[b]
4ws	*In(1)*$w^{m4L}w^{m4R}$	*In(1)*w^{m4},$w^{m4}w^{1}$ */ Df(YS)bb Su(var)*	BL 3805

[a] BL, Bloomington stock number; MA, former Mid-America stock number.
[b] Originally from the collection of I. Oster, possibly from H. J. Muller collection.
[c] Derivation described in materials and methods.

The number of *X/0* descendents varied from 10 to 65 for the different F_2 males.

To determine if any of the F_2 males were recombinant for elements affecting the inactivation level of *rst*, the numbers of eyes with and without rough patches in the sample of *X/0* descendents of each F_2 male were compared separately to the corresponding numbers for a sample of 74 *4LMcR/0* and of 60 *4r/0* males by χ^2 tests of 2×2 contingency tables. F_2 males whose *X/0* descendents differed significantly ($P < 0.05$) in *rst* inactivation from the *X/0* descendents of the parent chromosome with the same right inversion breakpoint were used to generate a second independent sample of *X/0* descendents that was retested. For only one F_2 male were both samples significantly different from the parental sample with the same right inversion breakpoint.

Genomic DNA analysis: Genomic DNA was prepared, and Southern analysis was performed according to standard protocols. Probes were made using the Random Primers DNA labeling system (GIBCO BRL, Gaithersburg, MD) and the clones λml.2 (Levis *et al.* 1982), λM407 (Goldberg *et al.* 1982), and pWM4. The plasmid pWM4 was provided by John Locke and contains a 3.8-kb *Eco*RI fragment from coordinates −29 to −25 kb near the *w* gene that was subcloned from λM505 (Goldberg *et al.* 1982) into pT7T3-19U (Pharmacia, Piscataway, NJ).

Fluorescent *in situ* hybridization (FISH): w^m/Y males were crossed to Amherst (wild-type) females. Brains from F_1 heterozygous female third instar larvae were dissected, hybridized, and detected according to Csink and Henikoff (1996), except that brains were treated with 0.5% sodium citrate for 10 min prior to fixing. The type I repeat probe was an 853-bp *Bam*HI fragment from the 3′ end of the type I element (Jakubczak *et al.* 1992). The 28S probe was made by polymerase chain reaction using the primers 5′-TTCAGGATACCTTCGGGACC-3′ and 5′-ATTTTCGCTTTCGCCTTGA-3′ to amplify Amherst genomic DNA according to standard protocols. These two probes were digested with a series of 4-cutter restriction endonucleases to yield an average size of 50–150 bp. The type I and 28S probes were end labeled with biotin-16-dUTP and with rhodamine-4-dUTP, respectively, using terminal deoxynucleotidyl transferase (GIBCO BRL) according to the manufacturer's instructions. The 359-bp repeat probe was made by polymerase chain reaction using the primers 5′-TACGAGCT CAGTGAGATATG-3′ and 5′-TTTCCAATCAAACTGTGTTC-3′ to amplify Amherst genomic DNA in the presence of 40 μm digoxigenin-11-dUTP; 80 μm dTTP; and 160 μm dCTP, dATP, and dGTP. Specificity of probes was confirmed by comparison to published maps (Appels and Hilliker 1982; Hilliker and Appels 1982).

Heterozygous $w^m/+$ nuclei were visualized using an Axiovert microscope (Zeiss, Thornwood, NY) and Deltavision 2.10 software (Applied Precision) with a cooled CCD camera (Photometrics, Tucson, AZ) at ×1600 magnification. Quantitation of hybridization signal in calibrated images was made by measuring the integrated intensity in a polygon surrounding each signal and then subtracting the integrated intensity in the same polygon moved onto the background. Computations and unpaired *t*-tests were performed using Statview 4.5 statistical software.

Reversion analysis: Males from stock *4T* were mutagenized with X rays (3000 R) and crossed to w^{1118} females. An estimated 20,600 heterozygous F_1 females were screened for individuals whose eyes had no unpigmented patches, *i.e.*, had either wild-type pigmentation or had only individual unpigmented ommatidia ("peppered"). Lines were established from 913 females by allowing them to mate with their w^{1118}/Y siblings. The phenotype was heritable in 96 of these lines of which 81 survived, and 25 proved to be hemizygously viable, male-fertile *X*-linked revertants, yielding a reversion rate of 0.12%. Males from these 25 lines were crossed to w^{m4} (stock *4Ta*) females, and salivary glands were dissected from the F_1 heterozygous third instar larvae for polytene cytology as previously described (Talbert *et al.* 1994). All 25 *4T* derivatives were eventually found to have secondary rearrangements, although 4 were initially misidentified as unrearranged from polytene analysis of revertant chromosomes heterozygous with w^{m4}. FISH analysis using the type I probe revealed that three of these four chromosomes had three distinct blocks of type I repeats, indicating that they had undergone secondary rearrangements. Subsequent polytene analysis in homozygotes revealed rearrangements in all four chromosomes that had been missed previously due to unfavorable positioning of chromosomes or to breakage of underpolytenized heterochromatin and somatic pairing of chromosome fragments to w^{m4}.

RESULTS

In a simple version of the oozing model for heterochromatic gene inactivation, heterochromatization proceeds continuously along the chromosome so that the frequency of inactivation of a gene near heterochromatin would be expected to depend on its distance from the nearest initiator of heterochromatin formation. Since the location of these initiators in heterochromatin is unknown, the distance between a reporter gene and the nearest initiator cannot be determined. This problem can be circumvented, however, by using two reporter genes expressed in the same cells. In cells in which the reporter gene that is closer to heterochromatin has been inactivated by heterochromatization, the probability of inactivating the more distal gene should depend on the distance between the two reporters.

We tested this prediction by examining the inactivation of the *w* and *rst* genes in stocks of w^{m4} and w^{mMc} flies. Both *w* and *rst* are expressed in the same third instar larval cells in the eye imaginal disc on either side of the morphogenetic furrow (U. Bhadra, M. Pal-Bhadra and J. A. Birchler, personal communication; Ramos *et al.* 1993). In both the w^{m4} and w^{mMc} inversions, the *w* gene is closer to heterochromatic sequences than the *rst* gene. The distance between the two genes was found to be 179 kb in a *y; cn bw sp* stock (Davis and Judd 1995), and is presumed to be identical to this or nearly so in the two inversions. Thus in flies carrying either inversion, cells in which *w* has been inactivated should have the same probability of also having *rst* inactivated. Tartof *et al.* (1984) reported that inactivation of *w* in w^{mMc} flies is weak, showing occasional unpigmented ommatidia (peppered), compared with that in w^{m4} flies, which have large patches of unpigmented ommatidia ("sectored"). If the probability of inactivating *rst* in w^- cells is the same in both genotypes, the greater inactivation of *w* in w^{m4} flies should be correlated with a greater inactivation of *rst* than in w^{mMc}.

Stocks *McT* and *4Ta* are descended from those used by Tartof *et al.* (1984) and have the expected phenotypes for *w* inactivation. Neither stock exhibits *rst* inacti-

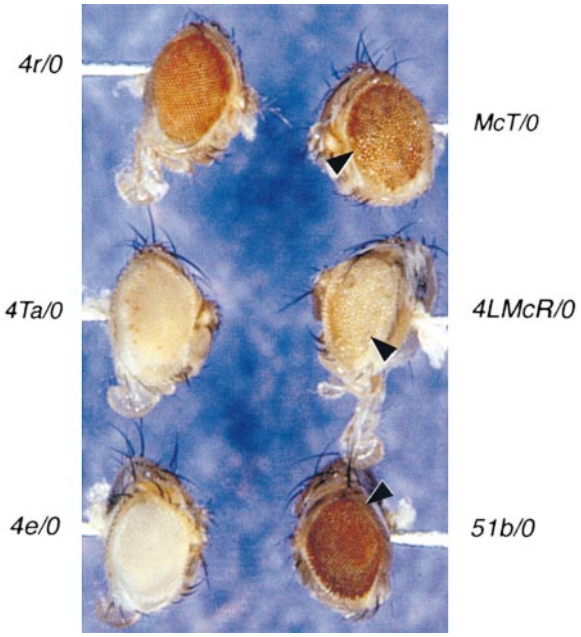

Figure 3.—Variegated eye phenotypes of *X/0* males. Note the rough patches in the eyes of the *McT/0*, *4LMcR/0*, and *51b/0* flies (arrowheads).

vation in euploid animals. PEV can be enhanced by genetically removing the *Y* chromosome to make *X/0* males (Gowen and Gay 1933). The suppressing effect of the *Y* chromosome on PEV is widely interpreted as a titration of heterochromatin-binding proteins by the heterochromatic sequences of the *Y* (Dimitri and Pisano 1989). To compare *rst* inactivation in the *McT* and *4Ta* stocks, we generated *McT/0* and *4Ta/0* males by crossing *X/Y* males of each stock to *C(1)M4/0* females. *McT/0* males had larger and more frequent rst^- patches than their less pigmented *4Ta/0* counterparts (Figure 3), indicating that the probability of inactivating *rst* in w^- cells is much higher in *McT/0* males. These results are consistent with those of Sinclair *et al.* (1989), who reported that $w^{mMc}/0$ males exhibit extreme *rst* variegation compared with less pigmented $w^{m4}/0$ males. These observations imply that other factors besides the distance from the heterochromatized *w* gene play significant roles in the spreading of inactivation to *rst*.

Frequency of inactivating *rst* in w^- cells is rearrangement dependent: The difference in the probability of inactivating *rst* in w^- cells of *McT/0* and *4Ta/0* males could be due to differences in the sequences linked to each inversion or to unlinked differences in the genetic backgrounds of the stocks. To address whether the difference is rearrangement linked, we sought other stocks with w^{m4} and w^{mMc} chromosomes that were likely to differ in genetic background.

We examined seven other w^{m4} stocks that ranged in eye phenotype from very weak inactivation of *w* to very strong. None had rough eyes in euploid males or females. The only other stock of w^{mMc} available, stock *McB*, is the immediate ancestor of stock *McT*. Since properties of the w^{mMc} chromosomes in these two stocks proved to be indistinguishable by molecular, cytological, and genetic criteria (data not shown), *McB* is not considered further. The *4LMcR* stock carries a $w^{m4L}w^{mMcR}$ recombinant chromosome and has a moderately strong degree of *w* inactivation. The stock was part of the collection of Muller's student I. Oster, and it probably derives from Muller's experiment showing the equivalency of the left ends of w^{m4} and w^{mMc} (Muller 1946). Because of this equivalency, this stock effectively is another w^{Mc} stock. A few (2–3%) euploid flies of this stock have patches of rough eye tissue. The phenotype of this stock more closely matches Muller's (1946) original description of w^{mMc} than does that of the *McT* stock. We also examined a stock of w^{m51b}, which has weak inactivation of *w* (Tartof *et al.* 1984; Sinclair *et al.* 1989; Spofford and DeSalle 1991). Our w^{m51b} stock (stock *51b*) did not exhibit the inactivation of *rst* in euploid animals that was noted by W. K. Baker (Lindsley and Zimm 1992).

For all of these w^m stocks, the amount of extractable pteridines was measured in both *X/X* females and *X/0* males as an indicator of w^+ activity. Although stock-specific differences in head size or pigment levels per cell could make pigment quantities an inadequate measure of variegation, visual ranking of stock phenotypes gave the same relative order of severity as did pigment assays, except that Amherst *X/X* females had no variegation but did not have the most pigment. The rst^+ activity in *X/0* males was measured as the percentage of eyes without rough patches. Scatter plots showing rst^+ activity in *X/0* males compared to pigments in *X/0* males and in *X/X* females are shown in Figure 4. The level of rst^+ activity in *X/0* males was higher for all of the w^{m4} stocks (45–99% of wild type), regardless of their level of w^+ activity, than in stocks *McT*, *4LMcR*, and *51b* (12–35%). This suggests that there are rearrangement-linked elements that make the probability of inactivating *rst* in w^- cells higher for w^{mMc} and w^{m51b} stocks than for w^{m4} stocks. No significant correlation was found between w^+ and rst^+ activities in *X/0* males (Figure 4A); however, this could be because pigment measurements are too insensitive to accurately detect differences among low levels of pteridines. When the rst^+ activity of *X/0* males is compared instead to the w^+ activity of the corresponding *X/X* females (Figure 4B), there is still no correlation, even when considering just the w^{m4} stocks.

"Skipping": Examination of the inactivation of *rst* in *X/0* males allowed us to test another prediction of the oozing model: that spreading should be continuous. If heterochromatization propagates continuously along the chromosome from the inversion breakpoint, it must first inactivate the more proximal *w* gene before it can inactivate the more distal *rst* gene. No pigmented rough eye tissue, in which heterochromatization has skipped the *w* gene, should therefore be observable. In contradiction to this expectation, large patches of rough eye

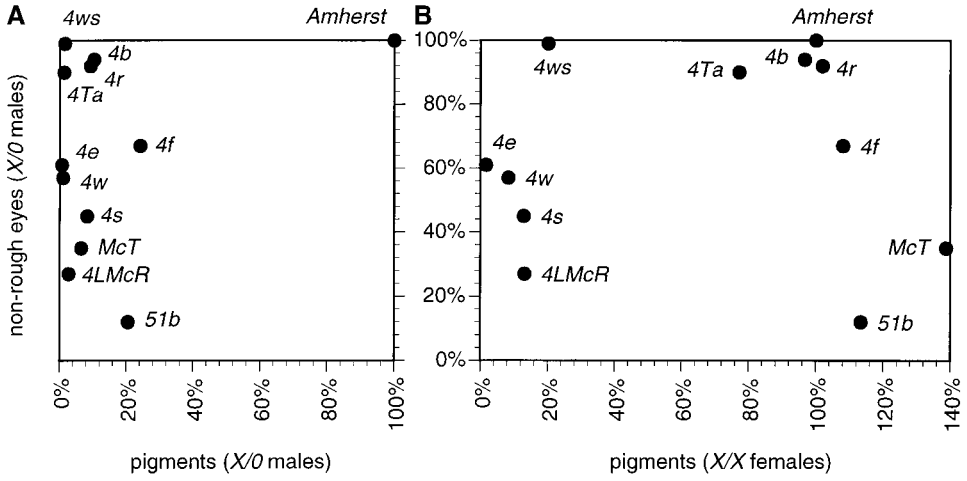

Figure 4.—Scatter plots of w^+ and rst^+ expression. (A) Pteridine measurements *vs.* nonrough eyes in *X/0* males, expressed as percentages of wild type. (B) Pteridine measurements of *X/X* females *vs.* nonrough eyes in *X/0* males.

tissue that included pigmented ommatidia (Figure 5) were observed in 18 out of 25 *4LMcR/0* males, suggesting that gene inactivation can spread discontinuously along the chromosome. Caution is necessary in interpreting this result, however, because the rough eye phenotype results from failure to order and eliminate surplus pigment cells during eye development, and is therefore an aggregate property of the local number and arrangement of cells (Wolff and Ready 1991). Thus, it is not possible to determine by visual inspection whether the pigmented cells within rough patches are actually inactivated for *rst*.

Figure 5.—The eye of a typical *4LMcR/0* male with mostly rough ommatidia. The arrowheads indicate pigmented (w^+) cells within the rough area.

Dominant modifiers: The rearrangement-linked element(s) that account for the difference in the probability of inactivating *rst* in w^- cells of w^{mMc} and w^{m4} flies can be mapped by recombination between these two inversions. The mapping might be obscured, however, if there are also differences between stocks in dominant modifiers of PEV that are segregating in the same cross. Dominant modifiers of PEV are relatively common, and an estimated 90% of them are not specific for individual variegating alleles (Talbert *et al.* 1994). Dominant modification can be brought about both by changes in the dosage of particular genes and by changes in the overall amount of heterochromatin (Spofford 1976). We tested for dominant modifiers in the w^m stocks using bw^{VDe2}, which responds to modifiers similarly to w^{m4} (Sass and Henikoff 1998). Wild-type males and males of each w^m stock were crossed to bw^{VDe2}/CyO; *st* females. Since bw^{VDe2} is dominant and the *w* and *st* eye color genes are haplosufficient loci, the amount of pigment present in $w^m/+$; $bw^{VDe2}/+$; $st/+$ female progeny should vary as a result of dominant modification of variegation at the *bw* eye color locus. These female progeny were classified into one of five phenotypic classes. Those with the least amount of pigment were assigned a value of 1 and those having the most were assigned a value of 5, so that a mean phenotypic rank for modification could be determined for each genotype. This mean rank can be used to compare the phenotypes of flies of each genotype, and therefore can describe the relative enhancement or suppression of *bw* variegation by dominant modifiers in each stock. A scatter plot of these mean ranks against the pigment values of homozygous females of each w^m stock is shown in Figure 6. It is apparent that the variation in pigment values among the different w^{m4} stocks

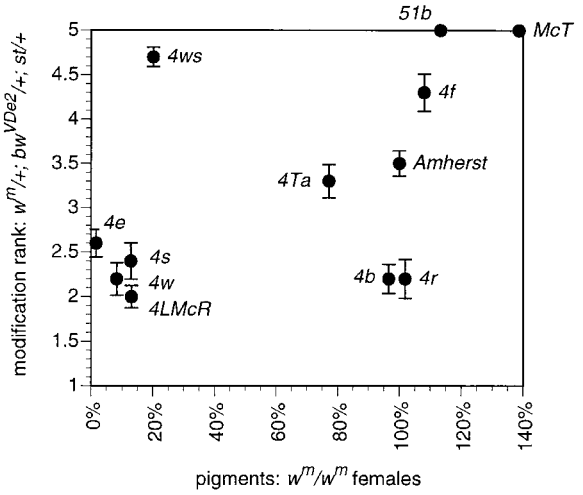

Figure 6.—Scatter plot of pteridine measurements of w^m females *vs.* mean ranks of the phenotypes of $w^m/+; bw^{VDe2}/+; st/+$ females.

is not explicable solely by the variation in dominant modifiers of PEV between stocks. The extreme suppression of bw^{VDe2} by *McT* relative to *4LMcR* is associated with extra heterochromatin in *McT* (discussed below).

Comparison of the $w^m/+; bw^{VDe2}/+; st/+$ females with their $+/Y; bw^{VDe2}/+; st/+$ brothers can give an indication of whether or not the dominant modifiers are linked to the *X* (or *Y*) chromosome. By this test, the suppression of $bw^{VDe2}/+$ in $McT/+$ females is *X*-linked and in $51b/+$ females is partly *X*-linked and partly autosomal (data not shown). *X*-linked differences in modification were not observed in the other stocks.

Mapping the rearrangement-linked elements: The *4LMcR* and *4r* stocks were used to map the rearrangement-linked elements(s) that affect the probability of inactivating *rst* in w^- cells. These stocks were chosen because they differ markedly in inactivation levels for both *w* (13% *vs.* 102% of wild-type pigments, respectively) and *rst* (27% nonrough eyes in $X/0$ males *vs.* 92%) and because they may have comparable genetic backgrounds, on the basis of their similar mean ranks for dominant modification of bw^{VDe2} (2.0 and 2.2).

If a rearrangement-linked element affecting the inactivation of *rst* in w^- cells is exchanged between *4r* and *4LMcR*, then the recombination event should be detectable as a change in the proportion of rough-eyed $X/0$ progeny. A male that has the right end of *4r* (w^{m4rR}) and is recombinant for the element should have an increased proportion of rough-eyed $X/0$ sons compared to those of $4r/0$ males. Conversely, a male that has the right end of *4LMcR* (w^{mMcR}) and is recombinant for the element should have a decreased proportion of rough-eyed $X/0$ sons compared to those of $4LMcR/0$ males. Recombination generated 36 w^{mMcR} F_2 males and 53 w^{m4rR} F_2 males. Only one recombinant, in two rounds of testing, generated $X/0$ progeny with a distribution of rough and nonrough eyes that differed significantly ($P < 0.001$) from the expected distribution based on the right inversion breakpoint it received: $w^{m4rR\text{-}126}/0$ males had 153/202 nonrough eyes (76%) compared with 110/120 (92%) for $4r/0$. This difference may be explained as the recombination of a rearrangement-linked element found ~1–2 map units from *w* at the right end of the chromosome. It is unlikely, however, that this element accounts for more than a small part of the difference between $4LMcR/0$ and $4r/0$ males (27% nonrough eyes *vs.* 92%, respectively). This implies that at least one other rearrangement-linked element affecting *rst* inactivation is located within ~1 map unit of the *w* gene, either in the euchromatic *w-rst* region or in the adjacent heterochromatin.

Quantities of major satellite repeats: Possible candidates for this rearrangement-linked element include the quantities of the repeats found in the proximal heterochromatin of the w^m inversions. To measure the quantities of the major repeats, FISH was performed on mitotic figures from $w^m/+$ larval neuroblasts using probes for the type I repeats, the 28S rDNA, and the 359-bp repeats (1.688 satellite). To compare the hybridization signals between genotypes and control for cell-to-cell variation in hybridization efficiency, the quantity of signal on either end of the w^m chromosome was normalized to that on the wild-type control chromosome in the same mitotic figure for each probe. Measurements were made on five to nine different mitotic figures of each genotype, and mean values were calculated. Signals from the right end of each inversion were compared to look for effects of the quantities of repeats in the *cis*-block of heterochromatin on the inactivation of *w* and *rst*. In addition, the total chromosomal signals (right plus left ends) were compared to look for overall titration effects of different quantities of heterochromatin (Figure 7).

Five different w^{m4} stocks were examined, ranging in phenotype from strongly (*4e*) to weakly (*4r*) inactivated for *w*. The mean values for total type I repeats ranged from 98 to 137% of wild type. *4r* had significantly more type I repeats than *4f* and *4Ta*, but other pairs of w^{m4} stocks were not significantly different from each other. *4r* differed from *4Ta* at the right end, and from *4f* at the left. A total of 52–60% of the type I signal was found at the right end, somewhat less than the 66% previously reported (Appels and Hilliker 1982). The mean values for total 28S repeats in the w^{m4} stocks ranged from 69 to 94% of wild type. The reduced amounts of signal from 28S repeats relative to wild type suggest that w^{m4} chromosomes may have less total heterochromatin than wild type, as noted by Spofford and DeSalle (1978). A total of 93–96% of the 28S signal was found at the right end, in reasonable agreement with the 98% previously reported (Appels and Hilliker 1982). *4r* had significantly more 28S repeats at the right end than *4f* and *4h*, but other pairs of w^{m4} stocks did not differ significantly. The mean values for 359-bp repeats in these

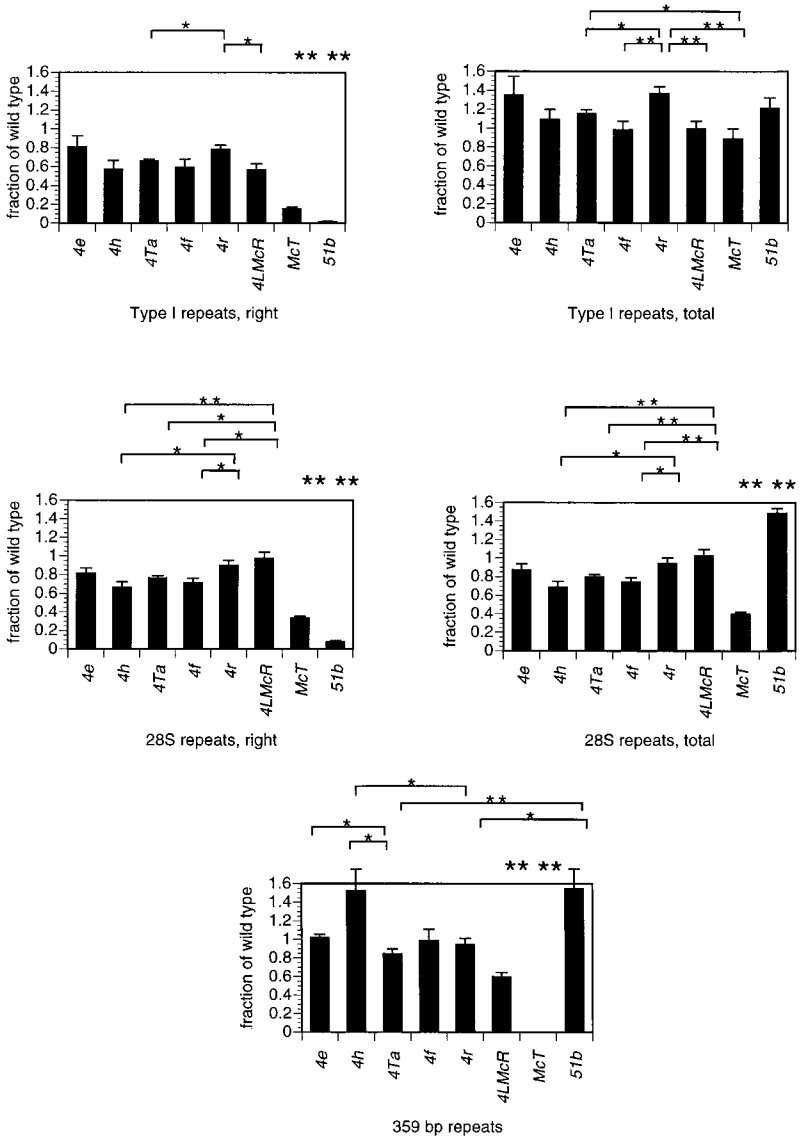

Figure 7.—Quantities of major heterochromatic repeats on w^m chromosomes. Means and standard errors for the fraction of repeats on a chromosome relative to the wild-type (Amherst) homolog in $w^m/+$ larval neuroblasts. Brackets connect chromosomes with significantly different means. * $P < 0.05$; ** $P < 0.01$. Two large asterisks indicate that the stock mean is significantly different from all other stock means with $P < 0.01$, except for *4LMcR vs. 4f*: $P = 0.015$.

stocks ranged from 84 to 153% of wild type. *4h* had significantly more 359-bp repeats than *4r* and *4Ta*, and *4e* also had more than *4Ta*.

Overall, the w^{m4} chromosomes appear rather similar to each other, and the relevance of the measured differences in repeats for phenotypic differences between stocks is unclear. It should be noted that the weakly inactivated *4r* stock does not differ significantly in repeats from the strongly inactivated *4e* stock. Since an increased dosage of the NO suppresses *w* inactivation, the higher amounts of type I and 28S repeats seen in *4r* relative to some other stocks might help explain the low level of *w* inactivation seen in this stock. If so, it is interesting that this increased dosage, which presumably acts *in trans* (Spofford and DeSalle 1991), does not appear to suppress bw^{VDe2} (Figure 6). The differences between stocks in the number of 359-bp repeats do not correlate with phenotype in any obvious way. The large standard error for *4h* adds further doubt to the phenotypic relevance of the measured differences in 359-bp repeats.

Among the *4LMcR*, *McT*, and *51b* chromosomes, significant differences from the w^{m4} stocks in the quantities of the three major repeats were found easily, but none correlated with the frequent inactivation of *rst*. The total type I signal in *51b* did not differ significantly from any other stocks, while *4LMcR* and *McT* differed from only one or two, respectively, of the w^{m4} stocks. The amount of type I signal at the right end was similar to w^{m4} stocks in *4LMcR*, reduced in *McT*, and absent in *51b*. The total 28S signal was increased in *51b*, reduced in *McT*, and at wild-type levels in *4LMcR*. The 28S signal at the right end was strongly reduced in *51b* and *McT*, but was at wild-type levels (slightly increased relative to most w^{m4} stocks) in *4LMcR*. Compared with the w^{m4} stocks, 359-bp repeats were increased in *51b*, decreased in *4LMcR*,

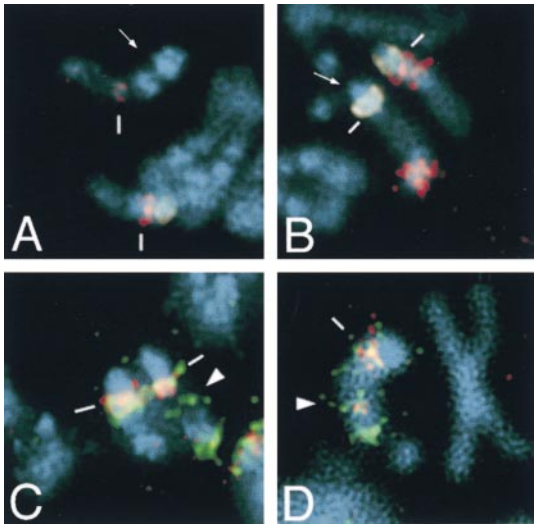

Figure 8.—FISH analysis of chromosomes. (A) *McT*/+. (B) *51b*/+. (C) $w^{m4hx537}$/+. (D) w^{m4Tx56}/+. Lines mark proximal rDNA. Arrows indicate anomalous heterochromatin not found on the wild-type *X* chromosome. Arrowheads mark heterochromatin contiguous with *w* in w^{m4} revertants. Blue, DAPI; pale amber, 359-bp repeats; red, 28S repeats; green, type I repeats. Magnification, ×2028.

and absent in *McT*. Thus, there is no consistent difference in the quantity of any of the three major repeat classes to which a high level of *rst* inactivation might be attributed.

Cytological examination revealed that the *McT* chromosome is actually an *X* · *YL* translocation with the point of exchange in the rDNA (Figure 8A). This observation was confirmed genetically by crossing *McT*/*Y* males to *C(1)DX, yf*/*0*; *T(Y;3)A78*/+ females and testing the male progeny for fertility. *McT*/*0; T(Y;3)A78*/+ males were fertile, while *McT*/*0;*+/+ males were sterile. In parallel crosses with *4Ta*/*Y* males, both classes of male progeny were sterile. Since *T(Y;3)A78* is deleted for *Y* chromosome bands *h1–h10*, which contain the fertility factors *kl-5*, *kl-3*, and *kl-2* (Gatti and Pimpinelli 1983), this indicates that the *McT* chromosome must carry these *YL* fertility factors. Despite the loss of much of the *X* heterochromatin, the presence of most of the *Y* in the *McT* chromosome clearly gives it a larger complement of heterochromatin than either the wild-type *X* or the other w^m chromosomes. The *51b* chromosome also has an increased heterochromatin content. Not only does it have higher than normal amounts of 28S and 359-bp repeats (Figure 7), but it also clearly has additional heterochromatin to the right of the 359-bp repeats compared with wild type (Figure 8B). Both *McT* and *51b* act as suppressors of bw^{VDe2} (Figure 6), as was previously reported (Lloyd *et al.* 1997). Comparison of inactivation levels of *w* and *rst* in *4LMcR* and *McT* stocks (Figure 3) suggests that the suppressing properties of *McT* (and probably *51b*) act on *w* and *rst* as well as *bw*.

Aside from the generalized suppressing effect of extra heterochromatin seen in *McT* and *51b*, and a possible more limited suppressing effect of the NO repeats seen in *4r*, gross changes in the major repeat classes in the w^m stocks were not predictive for the inactivation levels of either *w* or *rst*. It is possible that more subtle differences in heterochromatin that we were unable to detect may be important determinants of the inactivation of these genes.

Polymorphisms in the *w-rst* region: An alternative source of rearrangement-linked elements that could potentially affect *w* and *rst* inactivation is polymorphism in the euchromatic *w-rst* region. We probed for restriction fragment length polymorphisms (RFLPs) in the 25-kb region between *w* and the heterochromatic breakpoints in the w^m stocks (data not shown). The stocks could be placed into four distinct groups based on their *Hin*dIII restriction patterns near the *w* gene: (1) *4b, 4f, 4r*, and *4s*; (2) *4e, 4h, 4Ta, 4w*, and *4ws*; (3) *4LMcR* and *McT*; and (4) *51b*.

Do these RFLPs affect the inactivation of *w* and/or *rst*? It is suggestive that as a group the w^{m4} stocks with pattern 1 are more pigmented than those with pattern 2, although the two groups overlap in pigment levels. It is likely that among the w^m stocks several additional RFLPs could be found in the 179-kb region between *w* and *rst*. Unfortunately, the generation of recombinants for mapping phenotypes to particular RFLPs in this region is extremely improbable due to the proximity of heterochromatin. Instead, we attempted to map rearrangement-linked elements important for the inactivation of *w* and/or *rst* by comparing a closely related series of chromosomes.

Rearrangements in w^{m4} revertants: Revertants or partial revertants of a variegating rearrangement constitute a closely related series of chromosomes likely to differ only in elements affecting the inactivation of the variegating gene. Although the majority of revertants of variegating rearrangements are secondary rearrangements that separate the variegating gene from the bulk of the adjacent heterochromatin, Reuter *et al.* (1985) reported that 27% of w^{m4h} revertants lacked detectable differences in polytene cytology from their w^{m4h} parent. These reversion events were tightly linked to the w^{m4h} heterochromatic breakpoint. This class of revertants might include changes in the adjacent euchromatic region that affect *w* inactivation or changes internal to the heterochromatin that could help identify particular heterochromatic elements that are important for inactivation. We examined one of these revertants, $w^{m4hx537}$, and also investigated 25 *X*-linked viable male-fertile revertants of stock *4T* (see materials and methods) that had either wild-type pigmentation (full revertants) or had weak inactivation of *w* (partial revertants).

The reportedly unrearranged revertant $w^{m4hx537}$ was found to have a secondary inversion with breakpoints in proximal heterochromatin and *8CD*. All 25 *4T* derivatives were also found to have secondary rearrangements

with one break in proximal heterochromatin. Of these, 14 (6 full revertants and 8 partial revertants) had inversions with the second break at the site of the distal block of heterochromatin ("reinversions"). Of these, 10 were inversions with the second break in euchromatic sites. The 7 full revertants in this group had breaks at *5C*, *8A*, *8F* (twice), *9A*, *11B*, and *15CD*, while the 3 partial revertants had breaks at *9C*, *13C*, and *17A*. One full revertant was a transposition moving the chromosomal segment *12F–14A* to a position proximal to *3C* (new order: 1A-3C2|20het-14B|12E-3C2|14A-12F). Because no disruption of the region between *w* and the w^{m4} heterochromatic breakpoint was found by Southern analysis (data not shown), the transposed segment appears to be inserted into the proximal heterochromatin. Therefore, all of the secondary rearrangements in the *4T* revertants change the size of the block of heterochromatin adjacent to *w* and its distance from the remaining proximal heterochromatin.

Repeat organization of revertant chromosomes: We examined the secondary rearrangements of three *4T* revertants and of $w^{m4hx537}$ using FISH and determined which major repeats remain contiguous with *w* in these stocks. Type I repeats, but not 28S repeats, were detected adjacent to *w* in $w^{m4hx537}$ (Figure 8C) and in the revertant w^{m4Tx41}, which has its secondary euchromatic breakpoint at 11B. Both type I and 28S repeats were found to be contiguous with *w* in the partial revertants w^{m4Tx56} (Figure 8D) and $w^{m4Tx110}$, which have secondary euchromatic breakpoints at 13C and at 17A, respectively. $w^{m4hx537}$ and w^{m4Tx41} are very weakly mottled, whereas w^{m4Tx56} and $w^{m4Tx110}$ are more severely affected. The more severe phenotypes of w^{m4Tx56} and $w^{m4Tx110}$ may result from the greater amount of heterochromatin that is contiguous with *w* in these stocks, as indicated by the 28S hybridization signal.

We estimated the number of type I repeats contiguous with the *w* gene in $w^{m4hx537}$ by quantifying the type I hybridization signal in four $w^{m4hx537}/+$ females. The total type I signal on the $w^{m4hx537}$ chromosome was measured to be 112% of that on the wild-type chromosome, and 19% of it was found to be contiguous with the *w* gene. A typical wild-type haploid genome has ~250 type I repeats (Dawid *et al.* 1981), suggesting that there may be (0.19)(1.12)(250) = ~50 type I repeats adjacent to the *w* gene in $w^{m4hx537}$. Type I repeats vary in size and are interspersed with other repetitive elements (Dawid *et al.* 1981), but if the majority of repeats are similar in length to the full-length 5-kb element, this may represent >250 kb of heterochromatic sequence. Thus, nearly complete reversion of w^{m4} can occur while *w* is contiguous with a substantial heterochromatic block.

DISCUSSION

Rearrangement-linked differences in spreading are not explained by an oozing model: Schultz's (1939) characterization of heterochromatic inactivation of multiple linked genes as a process that spreads has influenced interpretation of this phenomenon for 60 years. The term "spreading" connotes a continuous and directional process of gene inactivation, properties that have been incorporated into the oozing model of Tartof *et al.* (1984). This model predicts that the frequency of the propagation of heterochromatic inactivation from one euchromatic gene to another should depend on the distance between the genes. The high frequency of inactivation of *rst* in w^- cells of w^{mMc} and w^{m51b} stocks relative to w^{m4} stocks is difficult to reconcile with this prediction.

The oozing model has been questioned previously on similar grounds. Clark and Chovnick (1986) found that $In(3R)ry^{11136}$ exerted heterochromatic position effects on both the more proximal *snake* and the more distal *piccolo* genes, while a similar inversion, $In(3R)ry^{54}$, had a weaker (unobservable) effect on *snake* than $In(3R)ry^{11136}$ but exerted a stronger effect on *piccolo*. The relative effects of the $In(3R)ry^{11136}$ and $In(3R)ry^{54}$ inversions on the proximal and distal genes parallel those of w^{m4} and w^{mMc}, respectively. The challenge to the oozing model presented by the behavior of the *In(3R)ry* inversions has received little attention. The issue may have been obscured because the *snake* and *piccolo* genes are expressed in different tissues and their phenotypes are likely to be differentially sensitive to reduced gene expression (Dutton and Chovnick 1991), although these considerations do not actually explain why the two inversions have different silencing behavior. Our results reinforce those of Clark and Chovnick, and are based on genes that are expressed in the same cells. We also demonstrate that the allele-specific differential silencing effects of the w^m chromosomes can be genetically mapped to the region of the silenced genes.

One way to modify the oozing model to explain these results would be to invoke polymorphic "dams" in the euchromatin that act *in cis* to reduce the extent of oozing past particular sites in the chromatin fiber. A dam between *w* and *rst* on the w^{m4} chromosomes would explain their low frequency of *rst* inactivation in w^- cells. However, this solution fails to elucidate other examples of PEV that conflict with the oozing model. For example, classical PEV of a *bw* transgene can be altered through a full range of phenotypes by making changes at the site of the transgene, with no changes whatsoever between the transgene and heterochromatin 60 kb away (Sabl and Henikoff 1996).

Another possible explanation for differences among alleles is differential sensitivity of a gene's regulatory region to the silencing machinery. Although differential sensitivity of *w* or *rst* might account for the lack of correlation among alleles that we see (Figure 4), it is hard to reconcile this mechanism with the oozing model. That is, one would have to make the *ad hoc* assertion that each of several *w* or *rst* alleles in an unselected

set of w^{m4} chromosomes is differentially sensitive to the advancing heterochromatin.

Spreading appears to be discontinuous: Other challenges to the oozing model are examples of discontinuous spreading, such as discontinuous compaction of polytene chromosomes (Belyaeva and Zhimulev 1991). Our observation of skipping of the *w* gene in presumedly rst^- patches in the eyes of *4LMcR/0* flies is another example. However, this example is not conclusive for the discontinuity of spreading because we cannot be certain of the activation state of the *rst* gene in all cells in a rough patch of tissue. It is important to note, however, that this ambiguity in interpretation also applies to previous studies of spreading from *rst* to *w* (Demerec and Slizynska 1937) and from *N* to *w* (Schultz 1939; Judd 1995), which constitute the sole genetic evidence underlying the hypothesis of continuous spreading. The eye patterning defects associated with inactivating both *rst* and *N* are multicellular phenotypes caused by changes in the number and arrangement of cells (Cagan and Ready 1989; Wolff and Ready 1991). Skipping of either *rst* or *N* in a subset of cells would be difficult to detect because it would require identifying w^-rst^+ or w^-N^+ cells in regions that are likely to have pattern disruptions caused by nearby rst^- or N^- cells. The failure to observe skipping of *rst* or *N* in an unspecified number of flies in these previous studies is therefore a negative result of uncertain significance. Our observation of skipping of *w* in 18 out of 25 *4LMcR/0* males, though inconclusive, is positive evidence for the view that spreading can be discontinuous. Because the activation state of *w* in any cell is unambiguous, the *w* gene is a more sensitive reporter for skipping. Had the earlier investigators of spreading looked for skipping of *w*, they likely would have concluded that spreading is discontinuous.

Quantity of major repeats does not correlate with *cis* inactivation: The mapping of the rearrangement-linked elements affecting the frequency of *rst* inactivation in w^- cells to the right ends of the inversions suggested that the quantity or quality of the proximal heterochromatin is important for the allele-specific effects; however, the quantities of the three major repeats of *X* heterochromatin in the w^m stocks did not correlate with the allele-specific inactivation levels of *rst* in w^- cells. Although Panshin (1938) inferred a correlation between the inactivation levels of *w* and the quantity of linked heterochromatin in derivatives of $T(1;4)w^{m11}$, his quantification was based solely on classical cytology. Using molecular mapping on deletion derivatives of $T(2;3)lt^{x13}\ P[ry^+w^+]$, Howe *et al.* (1995) failed to find a correlation between the inactivation of the *w* gene and the amount of linked *2L* heterochromatin, which is estimated to be on the order of 2.5 Mb for these derivatives. Howe *et al.* (1995) argued that the inactivation level must be influenced by the local heterochromatic environment. Our results are consistent with this latter interpretation. Each of the w^m chromosomes probably has in excess of 15 Mb of heterochromatin adjacent to *w*, and it is difficult to imagine how more than a small fraction of these sequences could have a direct effect on *w* and *rst* inactivation. On a more localized scale, the size of a block of repeats may be important for gene inactivation. Dorer and Henikoff (1997) found that the inactivation of a nearby essential locus by transgene arrays correlates with transgene copy number for arrays of 3–7 copies of a 10-kb transposon.

Increased total heterochromatin correlates with *trans*-acting suppression: The *McT* and *51b* chromosomes were found to carry anomalous extra heterochromatin and this correlated with *trans*-acting suppression of bw^{VDe2} by these two chromosomes. *Trans*-acting suppression of bw^{VDe2} has been reported previously in w^{mMc} and w^{m51b} stocks and was interpreted as a novel competition for heterochromatin-binding proteins between the two sets of rearrangement breakpoints (Lloyd *et al.* 1997). However, the presence of extra heterochromatin on these chromosomes argues for conventional suppression by increased amounts of total heterochromatin (Gowen and Gay 1933).

The *Y* chromosome material on the *McT* chromosome appears to have been acquired in a separate event from the original inversion, since it is absent in *4LMcR* and was also absent from a w^{mMc} stock available in the early 1980s (Hilliker and Appels 1982). The latter stock, which had reduced levels of rDNA, variegated for both *w* and *rst* in euploid animals matching Muller's (1946) description (A. Hilliker, personal communication). w^{m51b} also originally showed rough eyes in euploid animals (Lindsley and Zimm 1992), and the extra heterochromatin in the *McT* and *51b* stocks may have been selected because it suppresses the inactivation of *rst*, *N*, and other loci distal to *w*. Inactivation of this region is associated with reduced viability (Sinclair *et al.* 1989). It may be that w^{m4} stocks are able to tolerate having less heterochromatin than wild type (Spofford and DeSalle 1978) because inactivation does not spread to *rst* and other loci at a high frequency in these stocks.

Reversion of w^{m4} without gross rearrangement may be rare: We tried to find changes in the *X* heterochromatin or adjacent euchromatin that would affect *w* inactivation in linked revertants of w^{m4}, but all such events rearranged the *w* gene away from the bulk of adjacent heterochromatin. The frequency of nonrearranged reversion events reported by Reuter *et al.* (1985) is higher than has been reported in other reversion studies of PEV alleles (Dubinin 1936; Panshin 1938; Griffen and Stone 1940; Kaufmann 1942; Novitski 1961; Tartof *et al.* 1984; Pokholkova *et al.* 1993; Talbert *et al.* 1994; Henikoff *et al.* 1995). Rearrangements with one break in heterochromatin can sometimes be difficult to detect in polytene chromosomes, and we initially overlooked rearrangement in four of our revertants. Therefore, the true rate of PEV reversion events that delete or alter

determinants of inactivation near the euchromatin/heterochromatin boundary without gross rearrangement may be even lower than is suggested in earlier studies. Possible explanations for the rarity of these events are that the target region is small or that multiple regions must be simultaneously targeted to see an effect.

Partial reversion supports additive effects of heterochromatic repeats: Reversion of w^{m4} by secondary rearrangement has been variously interpreted in previous studies. The existence of nonvariegating complete revertants of w^{m4} with secondary rearrangements that leave at least 3 kb of type I sequence adjacent to the *w* locus has been taken as evidence that this adjoining heterochromatic sequence is not competent to silence *w* (Tartof *et al.* 1984, 1989). A similar conclusion was reached by Polholkova *et al.* (1993) for nonvariegating revertants of $T(1;2)dor^{var7}$, which retain at least 20 kb of *2R* heterochromatin adjacent to *dor*. In contrast, Reuter *et al.* (1985) argued that the rarity of revertants that did not variegate in the presence of enhancers (3/51) implied that a very short heterochromatic sequence, perhaps only a single type I element, is required to inactivate *w*, and that different levels of inactivation in partial revertants were evidence of additive effects of different numbers of tandem type I elements. The generation of partial revertants from other variegating rearrangements (Panshin 1938; Griffen and Stone 1940; Kaufmann 1942; Novitski 1961; Pokholkova *et al.* 1993) suggests that additive effects of heterochromatic repeats are general in PEV. This interpretation is supported by our finding that the more severe phenotypes of w^{m4Tx56} and $w^{m4Tx110}$ correlate with the greater amount of heterochromatin that is contiguous with *w* in these stocks, as compared to $w^{m4hx537}$ and w^{m4Tx41}. The estimated ~50 copies of type I repeats contiguous with *w* in $w^{m4hx537}$, which only shows inactivation of *w* when enhanced for PEV, suggest either that a fairly large number of type I repeats is necessary for strong inactivation, at least at polytene band 8CD, or that inactivation depends on another repeated sequence that is interspersed with type I repeats.

The coalescence model of spreading: An alternative model of PEV has been proposed that can explain the rearrangement-specific nature of spreading and its apparently discontinuous nature (Sabl and Henikoff 1996). In this model, heterochromatin formation is nucleated by pairing structures formed between repeated sequences. Natural heterochromatin is composed largely of highly repeated satellites and moderately repeated transposons that can potentially form complex pairing structures that attract heterochromatin-binding proteins. Genetic and cytological evidence from the megabase-sized bw^D heterochromatic insertion indicates that distantly linked heterochromatic regions physically associate or coalesce (Talbert *et al.* 1994; Henikoff *et al.* 1995; Csink and Henikoff 1996; Dernburg *et al.* 1996), and this looping coalescence is correlated with the strength of silencing of both a somatically paired bw^+ reporter gene and a nearby essential gene (Talbert *et al.* 1994). The inactivation of both genes is increased when bw^D is linked closely to pericentric heterochromatin and is decreased when the linkage is more distant. The coalescence of heterochromatic regions does not depend on homologous pairing of repeats, but may be a consequence of the "stickiness" of heterochromatized regions (Dorer and Henikoff 1997; A. K. Csink, personal communication). Gene inactivation may occur because coalescence mislocalizes genes to a nuclear region that is unfavorable for transcription.

In this model, spreading is explained by heterochromatic coalescence occurring on a smaller scale. Gene inactivation near heterochromatin is mediated by moderately repetitive elements that are scattered throughout the euchromatin and that can pair with nearby copies of themselves to form heterochromatin. Coalescence of these local repeats with nearby blocks of heterochromatin in this model is analogous to the proposed looping of weak *Polycomb* group response elements to nearby strong elements to explain the spreading of developmental silencing (Pirrotta and Rastelli 1994).

The results presented in this study are explicable in terms of this model. The polarity of spreading occurs because the probability that genes become associated with heterochromatin is larger for nearby genes than for more distant ones, so *w* is inactivated more frequently than *rst*. The model predicts that the local distribution of repetitive elements near the silenced genes determines to what extent spreading occurs in different rearrangements. Differences in the level of *w* inactivation should depend on the number and kind of repeated elements near the *w* gene. These elements might be detectable in part as the restriction pattern polymorphisms found near *w*. Repeated elements present between *w* and *rst* in the w^{mMc} and w^{m51b} stocks but absent in w^{m4} stocks might account for the higher level of *rst* inactivation in the former stocks. An additional possibility consistent with this model is that allele-specific differences in sensitivity of *w* or *rst* result from local differences in the association between a gene and heterochromatin (Sass and Henikoff 1999).

The model predicts that skipping of genes is possible because a repeated element near a more distant gene may become associated with heterochromatin through looping that allows a more proximal gene to escape inactivation. Skipping should be infrequent, though, because the proximal gene becomes constrained on both sides, increasing the chance that it also becomes associated with heterochromatin. Thus w^+ rst^- patches are less frequent than w^-rst^- or w^-rst^+ patches.

The predicted repetitive nature of the determinants of spreading makes reversion of PEV alleles difficult except by moving the affected genes away from large heterochromatic blocks, so that most reversion events are secondary rearrangements. The small block of het-

erochromatin contiguous with the w^+ gene in a secondarily rearranged w^{m4} revertant becomes less able to inactivate w^+ because it is more distant from the proximal *X* heterochromatin and coalesces with it less frequently. The greater the size of the contiguous heterochromatic block, the greater its probability of coalescing with the proximal *X* heterochromatin and inactivating the *w* gene.

In conclusion, our observations on the variable extent of spreading in different rearrangements point out serious shortcomings of oozing models. Furthermore, the continuity of spreading is called into question by our observation of apparent skipping and the realization that the traditional evidence for continuity is inherently inconclusive. Rather, our observations can be understood if the coalescence of nearby repetitive elements underlies the phenomenon of spreading.

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