Distance and Pairing Effects on the *brown*^{Dominant} Heterochromatic Element in Drosophila

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ABSTRACT

We examined the behavior of the *brown*^{Dominant} (*bw*^{D}) heterochromatic insertion moved to different locations relative to centric heterochromatin. Effects were measured as the degree of silencing of a wild-type *brown* eye pigment gene by *bw*^{D} across a tandem duplication. A series of X-ray-induced effects were recovered at high frequency. *Cis*-acting enhancers were obtained by relocation of the duplication closer to autosomal heterochromatin. Enhancers were also recovered on the homologous chromosome when it was similarly rearranged, revealing a novel interhomologue effect whereby interactions occur between genetic elements near opposite ends of a chromosome arm rather than between paired alleles. *Cis*-acting suppressors were obtained as secondary rearrangements in which the duplication was moved farther away from heterochromatin. Suppression was correlated with loss of cytological association between *bw*^{D} and the polytene chromocenter. Surprisingly, the distance from *bw*^{D} to the chromocenter was not correlated with the strength of enhancement or suppression. We propose that *bw*^{D} fails to coalesce with the chromocenter when its position along the chromosome places it beyond a threshold distance from heterochromatin, and this threshold depends upon the configuration of both the chromosome carrying *bw*^{D} and its paired homologue.

A conspicuous feature of higher eukaryotic chromosomes is the distinction between euchromatin and heterochromatin. Most genes lie in euchromatin, which disperses at interphase, whereas most tandemly repeated sequences lie in heterochromatin, which remains condensed. The molecular basis for this cytological distinction is not well understood.

In Drosophila, the study of heterochromatin and its distinction from euchromatin is highly advanced (for a review, see GATTI and PIMPINELLI 1992). In large part, this is because position-effect variegation (PEV) provides a powerful genetic tool for studying this distinction. In PEV, euchromatic genes are frequently silenced when juxtaposed to heterochromatin, and heterochromatic genes can display comparable silencing when juxtaposed to distal euchromatin (reviewed by SPOFFORD 1976; HENIKOFF 1990). Studies of genetic suppressors or enhancers of these silencing effects can reveal components necessary for distinguishing euchromatin and heterochromatin. Among these components are genes that appear to encode *trans*-acting factors, such as *Su(var)* and *E(var)* loci (reviewed by EISSENBERG 1989; GRIGLIATTI 1991; REUTER and SPIERER 1992). In addition, the study of PEV also has led to the identification of *cis*-acting determinants of the heterochromatic state, such as the tandemly repetitive nature of sequences (DORER and HENIKOFF 1994) and the relative location of heterochromatic blocks along the chromosome (BAKER 1953; HESSLER 1958; WAKIMOTO and HEARN 1990; EBERL *et al.* 1993; TALBERT *et al.* 1994). Alterations affecting this latter determinant of the heterochromatic state, in which nearby blocks interact, will be referred to as heterochromatin distance effects. These effects might reflect the tendency for heterochromatin to fuse into a chromocenter, as seen most vividly in salivary gland polytene nuclei, and this fusion might be impeded by increasing the distance between heterochromatic blocks.

Heterochromatin distance effects were first established for genes normally located in pericentric heterochromatin. Studies of the heterochromatic *peach* gene of *D. virilis* (BAKER 1953) and *light* gene of *D. melanogaster* (HESSLER 1958; WAKIMOTO and HEARN 1990) showed that inversions and translocations to distal, as opposed to proximal, euchromatin cause PEV. These studies suggested that intervening euchromatin attenuates the expression of these genes, which require a heterochromatic environment to function. However, since these rearrangements also affected the size of the displaced heterochromatic block, it was difficult to determine how much of the effects seen were due to the distance between blocks and how much to the removal of flanking heterochromatin. In a study of the heterochromatic *rolled* gene of *D. melanogaster*, selection for reversion of *rolled* PEV led to examples in which an interstitial block of heterochromatin containing *rolled* was moved closer to pericentric heterochromatin (EBERL *et al.* 1993). This

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study established that both the size of an interstitial block and its distance from large blocks of heterochromatin (rather than to the centromere) were involved.

Most recently, a heterochromatin distance effect was detected for euchromatic gene silencing by PEV (TALBERT *et al.* 1994). In a study of the heterochromatic *brown*^{Dominant} (*bw*^{D}) element, which causes silencing of the euchromatic *brown* (*bw*$^{+}$) gene, a heterochromatin distance effect on *bw*$^{+}$ was found in both directions: stronger PEV occurred for translocations that moved the element closer to heterochromatin, and weaker PEV occurred for translocations that moved the element farther away from heterochromatin. Although these results with *bw*^{D} could generally be interpreted in terms of heterochromatin distance effects for autosomal rearrangements, translocations involving the *X* chromosome appeared to behave differently. *X* chromosome euchromatin caused suppression of *brown* PEV in a distance-independent manner.

A complication of the study of *bw*^{D} heterochromatin distance effects is that PEV was assayed as the effect of the heterochromatic element on a wild-type copy of the *brown* gene present on the homologous chromosome. This assay was necessary because the *bw*^{D} heterochromatic element is inserted within the *brown* gene coding region (Figure 1A), resulting in a null allele in *cis*. However, by basing the screen for modification of *bw*^{D} PEV on an interhomologue effect, we could not easily address the possibility that some of the translocations were selected because of their effects on pairing between homologues, rather than on the distance to heterochromatic blocks. To address this, we describe a system in which the *bw*^{D} element causes PEV on an adjacent copy of the *brown* gene present on a tandem duplication. We first present evidence that the basis for silencing is *para*-inactivation across paired copies of the duplication, as opposed to *cis*-spreading along the chromosome. We then report the use of this duplication in X-ray screens to recover modifiers that alter the distance of *bw*^{D} to blocks of heterochromatin. These modifiers were recovered at an extremely high rate, suggesting that this system will be of practical value both for testing potential mutagens and for recovering rearrangement breakpoints analogous to the transvection technique (LEWIS 1954; GELBART 1982; LEISERSON *et al.* 1994). The results of these screens, along with the cytological behavior of *bw*^{D} in polytene nuclei, suggest a model for heterochromatin distance effects involving both heterochromatic associations and homologue pairing.

MATERIALS AND METHODS

Fly stocks: Flies were raised in bottles or shell vials on standard corn meal-molasses medium or on instant food (Carolina Biological Supply). Except as noted, mutations are described by LINDSLEY and ZIMM (1992). *Su(bw*^{D}*)* chromosomes have been previously described (TALBERT *et al.* 1994). *Suppressor-of-Plum* or *Su(bw*^{V1}*)* is a misnomer, because this lesion is a tandem duplication of the *brown* gene and flanking sequences within 59E (HENIKOFF and DREESEN 1989; B. KADEL and T. R. F. WRIGHT, unpublished data), *i.e., Dp(2;2) 59E, bw*$^{+}$ *bw*$^{+}$. BYRON KADEL (unpublished data) recorded the isolation of a recombinant between *Dp(2;2) 59E, bw*$^{+}$ *bw*$^{+}$ and *bw*^{D} with variegated eyes, which he deduced to consist of the *bw*^{D} allele in the proximal site of the duplication and *bw*$^{+}$ in the distal site (see Figure 1B). He also constructed a recombinant with the *bw*^{1} null allele in the proximal site *[Dp(2;2) 59E, bw*^{1} *bw*$^{+}$*]*, which is used in this study. We refer to one of the *Dp(2;2) 59E, bw*^{D} *bw*$^{+}$ recombinants that we subsequently isolated as the *Byron* duplication, to honor Kadel's role in inspiring this study.

Isolation and characterization of *Byron*: We screened the speck progeny of a cross between *Dp(2;2) 59E, bw*^{1} *bw*$^{+}$ *sp/bw*^{D} *+; st* females and *bw*^{1} *sp; st* males for variegated pigmentation. These appeared at a frequency of 0.5%, and one such male was used to establish a line. *Byron sp/bw*^{D} *+; st* males were crossed to females carrying various *brown* alleles, and their progeny were scored and used for pigment assays.

X-ray mutagenesis screens: *Dp(2;2) 59E, bw*$^{+}$ *bw*$^{+}$ is associated with a semilethal mutation or is tightly linked to one, so that derivative stocks were maintained over the *CyO* balancer chromosome. However, because homozygous male escapers comprise ~10% of the offspring and are healthy and fertile, these were used for mutagenesis. Males were aged 2–3 days, exposed to a single 3000-r dose of X-rays and crossed to virgin females. Males were removed after 3–4 days and progeny were screened after aging for ≥3 days. Only flies showing altered pigmentation in both eyes were selected.

In the first screen, irradiated *Byron sp; st* males were crossed to *bw*^{D}*; st* females and their progeny were examined for eyes that displayed fewer wild-type spots than the intense orange-variegated eyes characteristic of *Byron/bw*^{D}*; st* flies. Candidate enhanced mutants were back-crossed to *bw*^{D}*; st* to check for heritability and then crossed to *bw*^{1}*; st*. Lines in which enhancement was seen when heterozygous for both *bw*^{D} and *bw*^{1} were examined further.

In the second set of screens, irradiated *bw*^{1}*; st* or *Df(2R)bw*^{5}*/CyO; st* males were crossed to *Byron sp; st* females, and their progeny were examined for eyes that displayed fewer wild-type spots than expected (see Figure 2). In addition, *bw*^{5}-bearing progeny with eyes that displayed more wild-type spots were also selected. Candidate enhanced and suppressed mutants were back-crossed to *Byron sp/CyO; st* to check for heritability and lines were established.

In the last screen, an *E(Byron)* chromosome designated *Nella* was mutagenized. The *Nella* chromosome, named in honor of NELLA KADEL, BYRON's daughter, who discovered *Su(bw*^{V1}*)* (LINDSLEY and ZIMM 1992), is a pericentric inversion derived from *Byron* with breakpoints at 39D and 57F. X-irradiated *Nella sp; st* males were crossed to *bw*^{1}*; st* females and their progeny were examined for eyes that showed stronger pigmentation than expected, where unaffected *Nella/bw*^{1}*; st* flies have slightly off-white eyes with pepper-and-salt *bw*$^{+}$ spots. Candidate suppressed mutants were back-crossed to *bw*^{1}*; st* to check for heritability and crossed to *bw*^{D}*; st* to confirm suppression.

Pigment assays: Flies were cultured in uncrowded bottles or vials on instant food at 25° and aged for ≥3 days. In some cases, the acidified ethanol macroscale method for drosopterin measurement was used, modified as described (HENIKOFF and DREESEN 1989). In other cases, a microscale procedure was adopted in which pigment from one to four heads was extracted in a volume of 10 μl and duplicate 3- to 5-μl samples in glass capillaries were measured for optical density in a magnifying spectrophotometric cell (Shimadzu). Except as

noted, control flies were *CyO/bw*^{1}*; st*, which have drosopterin levels that are ~70% of levels found in *bw*$^{+}$*; st* homozygotes.

Cytology: Salivary gland squashes were performed as described (TALBERT *et al.* 1994). Cytogenetic distances were based on the photographic representations of LEFEVRE (1974), except in Bridges' divisions 39–40, where the revised map of SORSA (1988) provided an unambiguous representation. Distances were measured in cm on the LEFEVRE map; for example, chromosome arm 2R euchromatin measures 42.5 cm from the junction with the chromocenter to the telomere. From a cross of *E(Byron) sp/bw*$^{-}$ *+; st* × *bw*^{1}*; st, E(Byron)*-bearing larvae were distinguished from their *bw*$^{-}$*; st* siblings by their pale yellow, as opposed to white Malpighian tubules. It was difficult to determine the precise locations of breakpoints in the 59D-F region, because of the overwhelming tendency for the *bw*^{D} element at 59E in linkage-enhanced chromosomes to associate with the chromocenter (see Table 3 and Figure 7) and the distortion of 59E caused by the *Su(bw*^{V1}*)* duplication (HENIKOFF and DREESEN 1989; B. KADEL, unpublished data). From a cross of *Su(Nella) + + sp/+ Bc Elp +; st/+* × *Nella + + sp/+ Bc Elp +; st/+, Su(Nella)/Nella* heterozygous larvae were distinguished from their *Bc*-bearing siblings by the absence of black cells. Breakpoint analysis in the 59E region also was impeded by the distortion caused by the duplication, although the association of *Nella* [an *E(Byron)*] with the chromocenter and the lack of association of *Su(Nella)* typically led to complete asynapsis and allowed *Su(Nella)* breakpoints to be adequately localized in most cases (*e.g.*, Figure 7).

RESULTS

***Para*-inactivation of *bw*$^{+}$ by *bw*^{D}:** The *bw*^{D} heterochromatic element consists of a large heterochromatic insertion into the *brown* gene at band 59E1–2 of an otherwise unrearranged chromosome (HINTON and GOODSMITH 1950; SLATIS 1955b) (see Figure 1). Because the insertion causes a null mutation of *brown*, PEV is only observable as the very strong dominant variegating effect on the *bw*$^{+}$ copy present on the homologous chromosome. This dominant effect is sufficiently strong and uniform that ethylmethanesulfonate-induced dominant suppressor mutations could be readily obtained (TALBERT *et al.* 1994). However, enhancer mutations might have been more difficult to detect in the context of that screen, given that *bw*^{D}/+ flies have only ~2% of normal red eye pigmentation (Table 1). To screen easily for enhancer mutations caused by linkage alteration of the *bw*^{D} element, a weaker variegating phenotype was desired. This requirement was met by generating a tandem duplication oriented centromere-*bw*^{D}-*bw*$^{+}$-telomere, designated *Byron* (Figure 1B), that displayed 10-fold higher levels of pigmentation than *bw*^{D} when heterozygous with the null *bw*^{1} point mutation (Table 1). *Byron* had the additional advantage over *bw*^{D} that PEV does not require pairing of homologous chromosomes.

At least two general models might account for the effect of *bw*^{D} on *bw*$^{+}$ in the *Byron* duplication. The traditional *cis*-spreading model for PEV asserts that the *bw*^{D} heterochromatic element induces an alteration in chromatin conformation characteristic of heterochromatin that spreads continuously through the heterochromatin-euchromatin junction into the gene on the tandemly duplicated copy. A second model, *para*-inactivation, asserts that a discontinous side-by-side interaction occurs between the *bw*^{D} heterochromatic element and the *bw*$^{+}$ gene (Figure 2). Possible mechanisms for *para*-inactivation would be the same as for *trans*-inactivation of *bw*$^{+}$, which is the silencing phenomenon responsible for the dominance of *brown* PEV alleles (HENIKOFF and DREESEN 1989).

By the *cis*-spreading model for PEV, there is no expected phenotypic effect of substituting a deletion allele for a null point mutation in the gene carried on the homologue, and this is generally the case for *brown*-variegating alleles (SLATIS 1955a). However, by the *para*-inactivation model, a deletion would be expected to cause more frequent gene silencing, since it removes the *bw trans* copy, which can potentially compete for

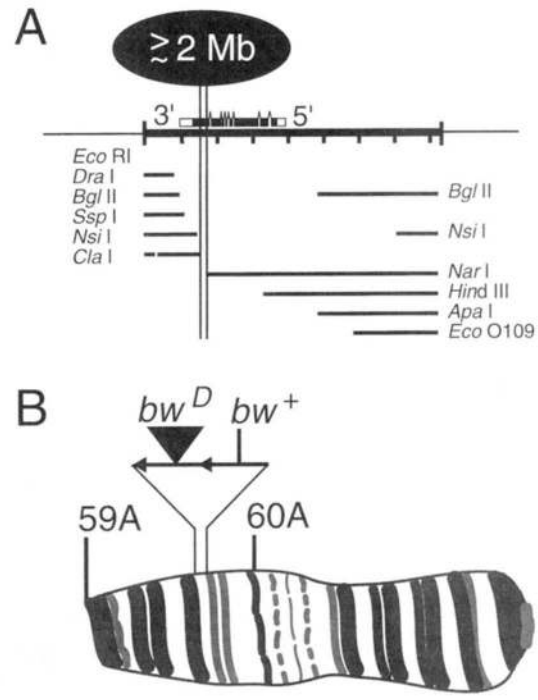

FIGURE 1.—(A) Structure of the *bw*^{D} insertion. Analysis on standard electrophoretic gels failed to detect restriction sites within the insertion (black oval); for each restriction endonuclease indicated, the expected band evidently migrated at limiting mobility (K. LOUGHNEY and J. M. JACKSON, unpublished data). Horizontal lines below the map represent bands observed when *bw*^{D} genomic DNA was digested with both *Eco*RI and the indicated endonuclease and probed with the 8373 bp *Eco*RI fragment (GenBank/EMBL Accession #L23543), indicated by the thick line. Pulsed-field gel analysis suggested the absence of *Eco*RI sites for ~1 Mb on either side of the insertion. Exons are depicted as boxes, coding regions are in black, and the scale below the thick line is in kb. (B) Schematic diagram of the *Byron* duplication inferred from genetic and cytological analysis (HENIKOFF and DREESEN 1989). The tip of polytene chromosome arm 2R is depicted, where the open triangle indicates the 59E region that is duplicated (tandem arrows) in *Dp(2;2) 59E*. The *bw*^{D} insertion (black triangle) is present on the proximal element of the duplication.

TABLE 1

Pigment levels in duplication-bearing heterozygotes

Genotype	Females (%)[a]	Males (%)[a]
$bw^D/+$	2	2
Byron/bw^1	12	23
Byron/*Df(2R)*bw^5	2	2
Byron/*Byron*	112[b]	113[b]
Byron/bw^D	12	13
bw^1-bw^+/bw^D	10	17
Byron/bw^+	71	89

[a] Percentages were calculated as the ratio of the mean of triplicate measurements for flies of the genotype indicated to the mean for control bw^+/bw^1; *st* flies × 100.

[b] Mutant spots were observed on a full red background (see Figure 2).

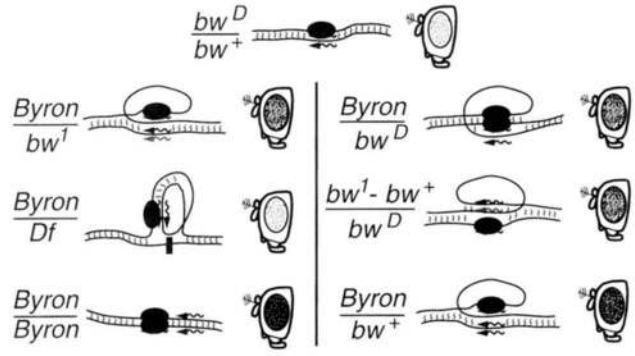

FIGURE 2.—Model for *para*-inactivation of the *brown* gene by the bw^D heterochromatic element. The bw^D insertion (black oval) interrupts the *brown* gene and *trans*-inactivates a paired bw^+ gene (black wavy arrow) on the homologous chromosome (bw^D/bw^+), where pairing between individual chromosomes (solid lines) is depicted as ladder rungs. Pairing between bw^D and bw^+ on a tandem duplication results in a silencing effect similar to that for *trans*-inactivation. Hypothesized paired configurations are shown for different combinations of bw^D, bw^+ and the null bw^1 mutation (grey wavy arrow), along with cartoons portraying the phenotypes. Orientation is proximal to the left and distal to the right.

side-by-side pairing with the *para* copy at the distal site (Figure 2). To test this possibility, we compared the phenotype of *Byron*/bw^1 to that of *Byron*/*Df(2R)*bw^5. A clear-cut difference was seen (Table 1) with enhancement by the bw^5 deletion, resulting in a phenotype similar to that of bw^D/bw^+ (~2% pigmentation). Further support for the *para*-inactivation model comes from examining other heteroallelic combinations. For example, *Byron*/*Byron* homozygotes are almost wild type, displaying more than additive levels of pigmentation, which is understandable if precise pairing of homologues in the homozygote almost completely eliminates side-by-side pairing of *para* copies. This is not explained by the *cis*-spreading model, which predicts additivity in homozygotes (SPOFFORD 1976). Furthermore, the equivalent effect of bw^D relative to bw^1 in *trans* to *Byron* (Figure 2) becomes understandable, since preferred pairing would occur between the copies of bw^D on both chromosomes, interfering with both *para*- and *trans*-inactivation (HENIKOFF and DREESEN 1989). We conclude that the *para*-inactivation model fully accounts for the *Byron* phenotype.

Enhancers of *Byron*: The *Byron* duplication provides a suitable genetic element for measuring heterochromatin distance effects, because rearrangements will move both the inducer of PEV and the target reporter gene as a unit. We screened for X-ray-induced enhancers of the *Byron* duplication by crossing mutagenized *Byron; st* males to bw^D; *st* females. The eyes of *Byron*/bw^D; *st* progeny are orangish with pepper-and-salt variegation and display a sexual dimorphism, with 12% pigmentation in females and 23% in males. Among ~10,000 progeny, two displayed increased pigmentation and 235 (~2%) displayed reduced pigmentation, from which 103 lines were established. The two suppressor lines were found to involve euchromatic breaks in 59E (where *brown* is located or close to it, see MATERIALS AND METHODS), perhaps within the bw^D element itself, thus reducing its *para*-inactivating effect. For 20 enhancer lines, the mutant effect was judged to be too subtle to score reliably. Of the remainder, enhancement was unlinked to the *Byron* duplication in 29 lines. For the other 52 lines (64%), enhancement showed linkage to *Byron*.

Polytene chromosome analysis revealed that all but seven enhancers involved visible breakpoints on the second chromosome. Five lines involved rearrangements with three or more breaks and are not considered further. In 17 lines, including the only two examples of *Y*-linkage, a rearrangement was seen with one breakpoint within 59E, and the other breakpoint was located in pericentric heterochromatin. It is possible that this high frequency of breaks in 59E resulted from the large size of the bw^D element, which appears to contain ≥2 Mb of simple sequence DNA (Figure 1A) and is visible during metaphase as a heterochromatic block (LINDSLEY and ZIMM 1992). Breaks within bw^D might result in new examples of PEV on *brown* and not linkage alterations of the intact *Byron* duplication. Indeed, pigmentation was patchy and relatively variable from eye to eye in 8 of the 17 lines, in contrast to the pepper-and-salt phenotype characteristic of bw^D and its linkage-modified derivatives. In addition, 4 of these 17 lines showed suppression as *E(Byron)*/bw^1 heterozygotes, in contrast to the enhancement seen for *E(Byron)*/bw^D heterozygotes in the screen. This behavior is understandable in terms of the *trans*-inactivation model (HENIKOFF and DREESEN 1989). A break that separates bw^D from bw^+ and places heterochromatin adjacent to bw^+ would behave similarly to a typical example of *brown* PEV, displaying weak *cis*-inactivation of bw^+ when heterozygous with bw^1 (seen as suppression) but subject to strong *trans*-inactivation by bw^D (seen as enhancement). Because these qualitative alterations in phenotype are suggestive of new posi-

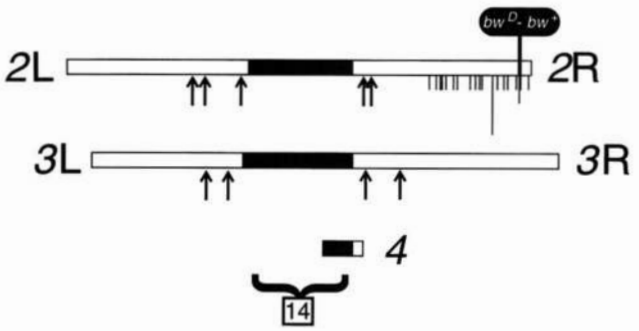

FIGURE 3.—Distribution of rearrangement breakpoints for two-break *E(Byron)*s. Distances within euchromatin (□) are shown to scale, measured from LEFEVRE's standard photographic representations of salivary gland polytene chromosomes (LEFEVRE 1974). Breakpoints very close to bw^D or in pericentric heterochromatin (■) were not resolved. First breakpoints are indicated as vertical lines of length in proportion to their frequencies (1–4 times). The cluster of four breaks proximal to bw^D is located within the exceptionally heavy band at 56F. Fourteen second breakpoints were in autosomal heterochromatin (bracket), and nine were in proximal euchromatin (arrows).

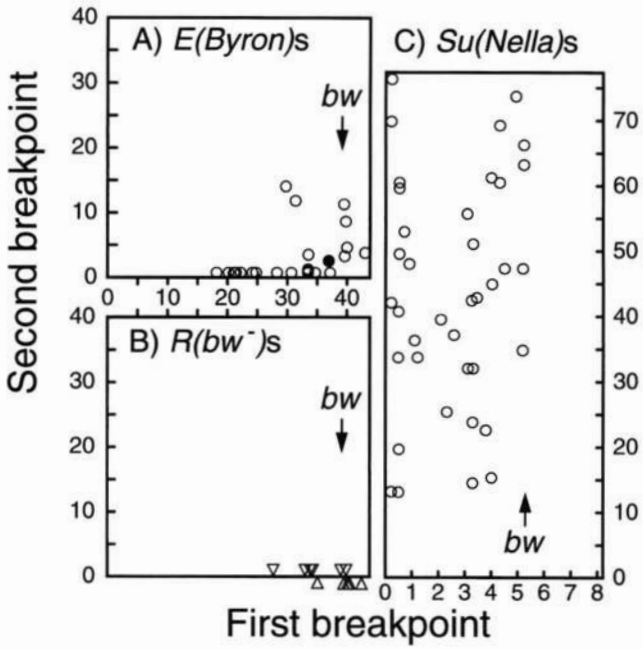

FIGURE 4.—Plots of breakpoint distributions for two-break rearrangements. (A) *E(Byron)*s, excluding those with one break very close to bw^D within 59E and the other break in heterochromatin, where ● indicates the *Nella* breakpoints. (B) $R(bw^-)$s, where ▽ indicates $R(bw^1)$s and △ indicates $R(bw^5)$s. All second breaks are in heterochromatin, which is zero on the Y-axis. (C) *Su(Nella)*s, excluding *T(Y;2)*s (note the different scale for the X-axis). Distances for all five long arms are measurements from base to tip in cm taken from LEFEVRE's photographic maps (LEFEVRE 1974).

tion effects on *brown*, the 17 lines are not necessarily informative with respect to possible linkage modification of bw^D.

In the 23 other lines, only two breaks were visible on salivary gland chromosomes. All were broken distal to 49F with a second breakpoint either in pericentric heterochromatin or in a proximal region of one of the long autosomal arms (Figures 3 and 4A). In 19 of these 23 lines, the first break was proximal to 59E. In two other lines, the first break was in the vicinity of 59E. In the remaining two lines, the first break was distal to 59E and the other break was in proximal 2R euchromatin. These 23 examples provided a test of the heterochromatin distance effect. In every case, the resulting rearrangement moved the bw^D heterochromatic element and the *brown* gene on the *Byron* duplication closer to heterochromatin. Although rearrangements involved all autosomal arms including chromosome *4*, no example of an *X*-linked rearrangement was found.

We asked whether the distance between the *Byron* duplication and heterochromatin correlates with the degree of enhancement. Pigment measurements were carried out on $E(Byron)/bw^1$; *st* flies of both sexes. As for $Byron/bw^1$; *st*, a sexual dimorphism was observed, with females typically showing more enhancement than males. However, no correlation with distance was detected (Figure 5A).

Enhancers of *Byron* on the homologous chromosome: The heterochromatin distance effect is not the only possible explanation for enhancement of *para*-inactivation in $E(Byron)/bw^-$ heterozygotes. An alternative is that somatic pairing of homologues is interrupted strongly in rearrangements that move the *Byron* duplication close to heterochromatin, leading to more frequent *para*-inactivation across the duplication. Analogous disruption of pairing is thought to underlie transvection (LEWIS 1954; GELBART 1982; LEISERSON *et al.* 1994). In support of this possibility, we note that the distribution of both first and second *E(Byron)* breakpoints is comparable to that obtained by GELBART (1982) for disruption of transvection at the *dpp* locus, which lies in a position on 2L comparably distal to that of *brown* on 2R.

These considerations led us to ask whether *para*-inactivation could be modified by linkage alterations involving the homologous chromosome. Accordingly, we irradiated males carrying a null bw^- allele, either bw^1/bw^1 or $Df(bw^5)/CyO$, and crossed them to *Byron/Byron* females, screening for enhancement in their progeny. Because $Df(bw^5)/Byron$ flies are strongly mutant (Figure 2), we screened these progeny for suppressors as well. In both screens, we obtained heritable enhancers linked to *brown* at a frequency of 0.75%. In the bw^1 screen, 18 heritable enhancers were selected for further characterization, and of these, 15 were linked to *brown*. In the $Df(bw^5)$ screen, 14 heritable enhancers were selected of which 13 were linked, and 5 heritable suppressors were selected, none of which was linked. Cytological analysis of 22 linked enhancers revealed the presence of rearrangements involving 2R in every case. Of these *rearrangements-of-*bw^- $[R(bw^-)]$s, 11 were found to have two-break rearrangements with one break in distal 2R

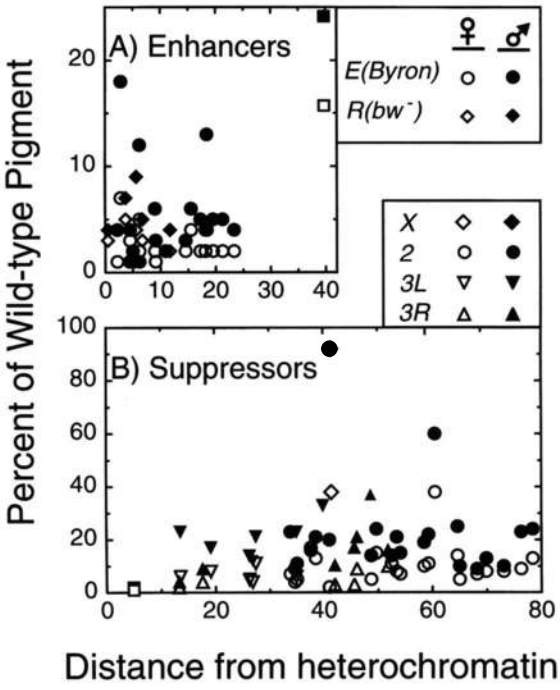

FIGURE 5.—Plots of pigment levels vs. distance from bw^D to pericentric heterochromatin for two-break rearrangements. (A) *E(Byron)/bw*^{1} (circles) and *R(bw*$^-$*)/Byron* (diamonds); (B) *Su(Nella)/bw*^{1} suppressors: translocation to *X* (diamonds), pericentric inversions (circles), translocations to *3*L (triangles down), and translocations to *3*R (triangles up). The comparable points for both *Byron/bw* in A and for *Nella/bw* in B are shown as boxes.

and a second break in heterochromatin (Figure 4B). The remaining 11 showed complex breaks and were not analyzed in detail.

It seemed possible that this striking *trans*-sensing effect (TARTOF and HENIKOFF 1991) associated with breaks in *trans* to the *Byron* duplication was caused by local disruption of homologous pairing. An example of such a local effect causing enhancement of *para*-inactivation is the reduced pigmentation seen for *Byron/bw*^{5} heterozygotes, whereby bw^5 removes the *trans* copy of the *brown* region that might otherwise compete with homologous copies on the duplication (Figure 2). Such local disruption of somatic pairing causing a more strongly mutant phenotype is similar to disruption of transvection (*i.e.*, pairing-dependent complementation) resulting from breaks proximal, but never distal, to sensitive heteroallelic combinations (LEWIS 1954; GELBART 1982; LEISERSON *et al.* 1994). This pairing-disruption model might be adapted to enhancement of *para*-inactivation, if it is asserted that local disruption of homologous pairing can also occur with breaks distal to *brown*, which account for 2 of the 23 *E(Byron)*s and 3 of the 11 *R(bw*$^-$*)*s. A test of this model would be to examine double heterozygotes in which both homologues are similarly rearranged, because this should approximately restore pairing. Such tests had been carried out by LEWIS (1954), GELBART (1982) and LEISERSON *et al.* (1994) in their demonstrations that disruption of transvection results from disruption of homologous pairing. For example, *dpp* heteroalleles with transvection-disrupting breakpoints proximal to *dpp* restored transvection, where the degree of restoration depended upon how closely the proximal breakpoints matched (GELBART 1982). Remarkably, *dpp* complementation occurred even when the second break was within the heterochromatin of different chromosomes. Similarly, if disruption of homologue pairing were responsible for enhancement of bw^D *para*-inactivation, then *E(Byron)/R(bw*^{1}*)* heterozygotes with approximately matched breakpoints should restore the original *Byron/bw*^{1} phenotype.

Four *E(Byron)*s with first breakpoints at 56F or 59E and four *R(bw*^{1}*)*s with first breakpoints near 56F or at 59F were tested in double heterozygous combinations. Regardless of first breakpoint position, nearly all *E(Byron)/R(bw*^{1}*)* double heterozygotes displayed *para*-inactivation phenotypes that were as strong or stronger than those observed for either *E(Byron)/bw*^{1} or *R(bw*^{1}*)/Byron* single heterozygous combinations (Table 2). This inability to restore the *Byron/bw*^{1} phenotype in double heterozygotes contrasts with the virtually complete restoration of transvection in comparable double heterozygotes (LEWIS 1954; GELBART 1982; LEISERSON *et al.* 1994). For example, the double heterozygote *T(2;3) E(Byron) 56F;80–81/T(2;3) R(bw*^{1}*) 57A;80,* with *2*R breakpoints only one Bridges' subdivision apart, showed more extreme *para*-inactivation than either single heterozygote, whereas all 36 *dpp* double heterozygotes tested with comparable breakpoints within 20 Bridges' subdivisions of one another showed nearly full complementation (GELBART 1982). We conclude that disruption of homologous pairing is not responsible for enhancement of *para*-inactivation.

Suppressors of *Nella*: If enhancement in *E(Byron)* heterozygotes occurs because of the reduced distance between bw^D and nearby heterochromatin, then moving the *Byron* duplication more distally should cause suppression. To test this, we irradiated flies carrying an *E(Byron)* rearrangement with euchromatic breakpoints favorable for cytogenetic analysis. This chromosome, called *Nella*, has breaks in 39D on *2*L and 57F on *2*R, resulting in an acrocentric configuration that places the bw^D element near the base of the short arm and 90% of the (continuous) second chromosome euchromatin on a very long arm (see Figure 6). The *Nella* second chromosome is associated with strong enhancement (<1% pigment) and is as viable as the *Byron*-bearing parent chromosome. Based on the heterochromatin distance effect model, we would expect selection for *Suppressors-of-Nella* [*Su(Nella)*s] to yield rearrangements, each with a proximal break in the short arm and a second break either near one of the tips of a normal long arm or scattered throughout the distal half of the very long arm of the *Nella* second chromosome. Since this distal half represents a larger target than the tips of the normal long arms, we might

TABLE 2

Pigment levels[a] in *E(Byron)/R(bw^1)* heterozygotes

	bw$^-$ derivatives:				
Byron derivatives	*bw*^{1}	*In(2R) 41;59F*	*T(2;3) 57A;80*	*In(2R) 41;56E*	*In(2) 40-41;57B*
Byron					
♀	++++	0	++	+	++
♂	++++	++	++	++	+++
In(2R) 43D;59E					
♀	+	0	+	++	+
♂	++	+	++	++	++
T(2;3) 56F;80-81					
♀	+	0	0	0	0
♂	++	0	+	0	0
T(2;3) 56F;78D					
♀	+	0	0	+	+
♂	+	++	+	++	+
T(2;4) 56F;100F					
♀	++	++	0	++	+
♂	++++	++	+	++	++

[a] 0, 0–1% pigment; +, 2–3%; ++, 4–7%; +++, 8–15%; ++++, 16–31%.

expect that a disproportionately large number of breakpoints would occur there.

We screened for X-ray-induced *Su(Nella)*s by crossing mutagenized *Nella sp; st* males to *bw^1; st* females. About 4500 progeny were screened, of which 156 (3.5%) displayed a suppressed phenotype. Among the 106 fertile survivors, 104 transmitted the mutation, from which 70 lines were established and mapped with respect to linkage. Of these, 68 (97%) showed linkage to the second chromosome, and 51 lines were subjected to polytene chromosome analysis. Only two mutations linked to chromosome *2* failed to reveal a second chromosome break by cytological or cosegregation analysis. Of the remainder, 38 involved two-break rearrangements (Figures 4C and 6). It is evident that all breakpoints are consistent with expectations of the heterochromatin distance effect model, moving the *Byron* duplication more distally. Pericentric inversions accounted for 22 of the 38 two-break rearrangements analyzed, suggesting that the very long second chromosome arm provided a very large target. Interestingly, the medial regions of chromosome arms *3*L and *3*R also provided targets, even though no breaks were seen in the medial region of *2*R.

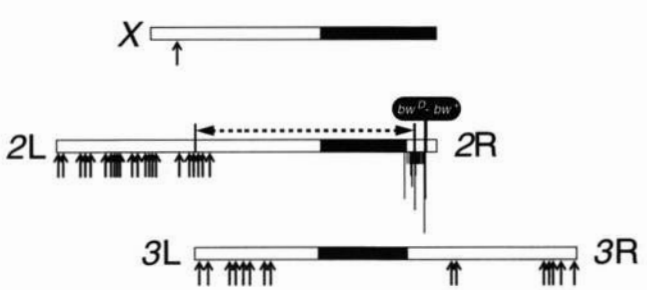

FIGURE 6.—Distribution of translocation and inversion breakpoints of *Su(Nella)*s. The *Nella* inversion (two-headed arrow) is *In(2LR)39D;57F*. Symbols are described as in the legend to Figure 3.

We also asked whether the distance between the *bw^D* element and heterochromatin correlates with the extent of suppression for each of the *Su(Nella)* lines. Pigment measurements were carried out on *Su(Nella)/bw^1; st* flies of both sexes. As for the *E(Byron)*s, there was a consistent sexual dimorphism, with males showing more pigment than females for all lines. Overall, no significant correlation with distance was detected, not even for the subset of lines with pericentric inversions (Figure 5B).

Cytological association of *bw^D* with the chromocenter: Previously, we reported that linkage-based enhancement of *bw^D*/+ was correlated with more frequent association of the *bw^D* heterochromatic element with the chromocenter of polytene salivary gland nuclei (TALBERT *et al.* 1994). In the present study, cytological analysis of *Su(Nella)/Nella* heterozygotes to determine breakpoint positions provided an opportunity to extend this observation to the *Byron* duplication in phenotypically suppressed and enhanced states within the same nucleus. Squashed nuclei from five different *Su(Nella)* rearrangements were scored for association of *bw^D* from *Nella* and *Su(Nella)* chromosomes with the chromocenter when both were clearly visible and asynapsed from one another. In >99% of these nuclei, the *Nella*-linked duplication was in contact with the chromocenter, whereas the *Su(Nella)*-linked duplication was not (Table 3 and Figure 7). This confirms our previous observation and directly demonstrates that linkage, rather than a diffusible factor, determines the association of *bw^D* with the chromocenter.

DISCUSSION

***Para*-inactivation:** The present analysis of heterochromatin distance effects was undertaken by first con-

TABLE 3

Association of the *Byron* duplication with the chromocenter in *Nella/Su(Nella)* heterozygotes

	No. of nuclei[a]
Total nuclei scored[b]	172
Asynaped	127 (72)
Nella associated, *Su(Nella)* not	126 (99)
Su(Nella) associated, *Nella* not	0
Both associated	0
Neither associated	1 (1)

[a] Values in parentheses are percentages.

[b] Combined results of squashes for five *Su(Nella)* rearrangements, *T(2;3)59D;68B, In(2)30E;59D, In(2)38B;59D, In(2)34C;59C, T(2;3)40F;61E,* superimposed on *In(2LR)38D;57F.* Only examples in which the *Byron* duplication on both chromosomes could be seen were scored. Squashes of ⩾20 other *Su(Nella)/Nella* heterozygotes revealed similar behavior without exception (data not shown).

structing the *Byron* tandem duplication of the *brown* gene region, in which one member of the duplication carries the bw^D heterochromatic element and the other member carries the bw^+ reporter gene. We provided evidence favoring a side-by-side interaction between these members of the duplication forming a loop structure and leading to silencing of bw^+ by bw^D (*para*-inactivation). *Para*-inactivation is analogous to a phenomenon described by GUBB and coworkers (1990). They provided evidence that cytologically visible tandem and inverted duplications of the *white* gene region displayed *zeste*-dependent repression attributable to loop or hairpin structures. Aberrations involving the homologous chromosome directly across from the duplications were shown to modify the repression associated with the tandem duplication as expected based on a pairing model. In the *para*-inactivation phenomenon described here, a small deficiency on the homologous chromosome directly across from the *Byron* duplication likewise modified the phenotype as expected based on a pairing model. Our findings support the generality of the pairing model proposed by GUBB and coworkers; whereas *zeste*-dependent repression of *white* and heterochromatin-dependent *trans*-inactivation of *brown* are quite different *trans*-sensing effects (TARTOF and HENIKOFF 1991), both appear to be similarly affected by altered chromosomal pairing configurations.

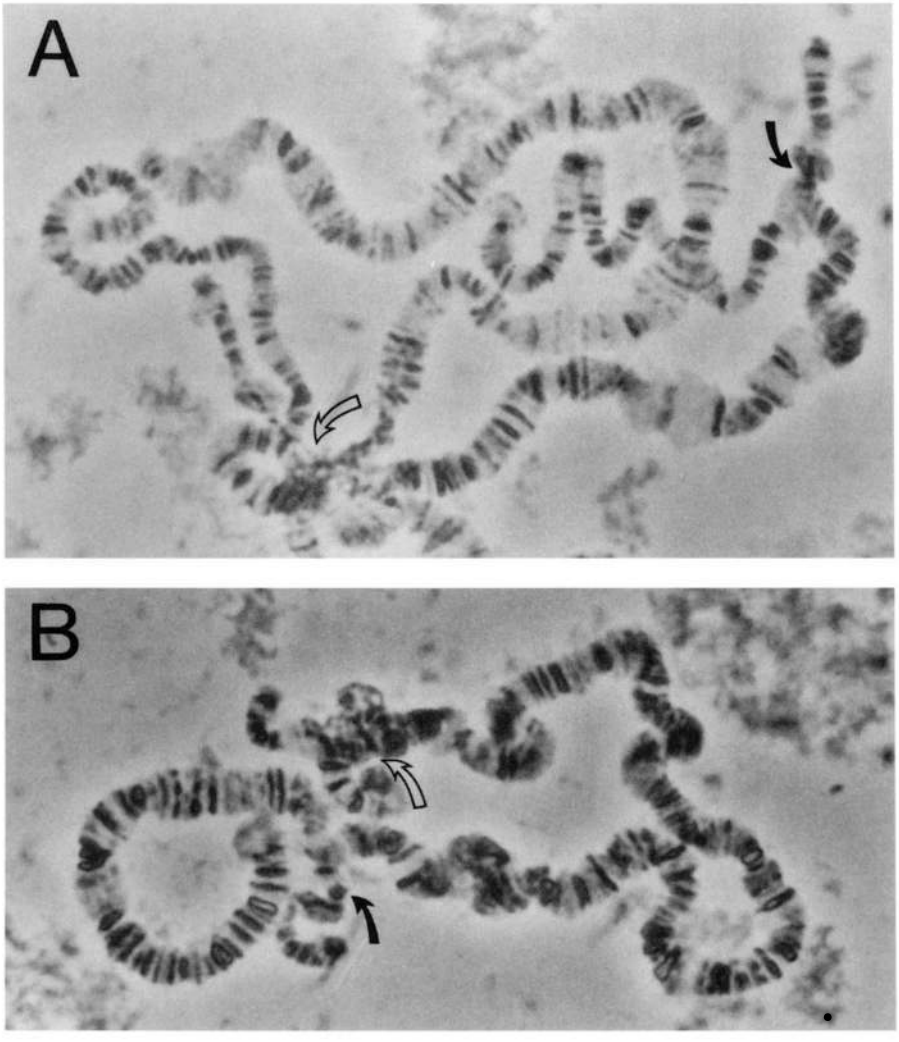

FIGURE 7.—Examples of salivary gland nuclei from *Nella/Su(Nella)* heterozygotes showing the typical behavior of the *Byron* duplications when squashing causes them to asynapse. In both cases, the *Nella* duplication (open arrow) is associated with the chromocenter, forming a loop, whereas the *Su(Nella)* duplication (filled arrow) is unassociated. *Su(Nella)* duplications are (A) *T(2;3) 59D;68B* and (B) *In(2) 59D;30E* superimposed on *In(2LR) 39D;57F.*

The basis for heterochromatin distance effects: The notion that the distance of a gene from centric heterochromatin influences PEV is an old one (DUBININ 1936; PANSHIN 1938; GRIFFEN and STONE 1940; KAUFMANN 1942). However, these early studies were difficult to interpret, because the same breakpoints that moved a block of heterochromatin to a distal location usually changed the size of the block. In subsequent studies, the movement of heterochromatic genes to euchromatin provided much stronger evidence for a heterochromatin distance effect, because here, PEV only occurred with movement to distal, not to proximal, euchromatin (HESSLER 1958; BAKER 1968; HILLIKER and HOLM 1975; WAKIMOTO and HEARN 1990; EBERL *et al.* 1993). In recent studies, the movement of heterochromatic blocks occurred without breaking within the blocks, thus allowing distance between heterochromatic blocks to be examined for blocks of the same size (EBERL *et al.* 1993; TALBERT *et al.* 1994). The isolation of *E(Byron)*s and *Su(Nella)*s confirms and extends these studies. We find that the distance effect accounts for all 23 enhancers consisting of two-break rearrangements, because these move the bw^D heterochromatic element closer to heterochromatin without breaking within the element. Conversely, all 38 suppressors consisting of two-break rearrangements moved the element farther away from heterochromatin. This distance effect is consistent with the idea that the tendency for heterochromatic blocks to coalesce into a chromocenter is reduced when a block is moved to a distal position (WAKIMOTO and HEARN 1990; EBERL *et al.* 1993), and this results in less frequent heterochromatic inactivation of the *brown* reporter gene (TALBERT *et al.* 1994).

The degree of enhancement in *E(Byron)*s and $R(bw^-)$s and of suppression in *Su(Nella)*s varies considerably from line to line, however no correlation with distance is seen (Figure 5). This is surprising; if a threshold distance for detection of breaks were simply caused by inability to select for weak enhancers or weak suppressors, then we would expect, for example, that the weakest enhancers found would coincide with the longest distances. This was not the case. All two-break *E(Byron)*s moved the *Byron* duplication closer to heterochromatin with both strong and weak enhancers at both ends of the distance scale. Likewise, all two-break *Su(Nella)*s moved the duplication farther away from heterochromatin without a correlation between distance and strength of suppression, not even for the *Su(Nella)* reinversions that span a distance equal to a long chromosome arm (circles in Figure 5B). To explain this, we assume that a small block of heterochromatin, such as bw^D, is weak in its heterochromatic effect when it is alone in the nucleus but is strong when it is part of a heterochromatic compartment or chromocenter consisting of heterochromatin from multiple chromosomes. Then the distance threshold can be understood as the periphery of a zone around the chromocenter within which the bw^D element is typically captured by the chromocenter and outside of which it typically escapes. Thus, when bw^D is moved away from the nearest block of heterochromatin but less than the threshold distance, it is consistently captured by the chromocenter and no distance effect is detected. However, when the threshold is exceeded, then bw^D escapes capture by the chromocenter and only a weak heterochromatic effect is seen. Variations in strength might result from variations in the local nuclear environment, and these might in turn depend on the spatial arrangement of chromosomes with respect to one another. The existence of a threshold is consistent with the observation that in >99% of *Nella/Su(Nella)* heterozygotes, in which the *Byron* duplications were unpaired in salivary gland squashes, the linkage-enhanced duplication was fused with the chromocenter and the linkage-suppressed duplication was not.

The results of the present study might help explain why about half of the $Su(bw^D)$s in our earlier study were associated with breaks scattered throughout the *X*-euchromatin, rather than being limited to the distal tip (TALBERT *et al.* 1994). One possibility we had considered was that the nucleolus encoded in *X*-heterochromatin interferes with looping of bw^D into the chromocenter (A. HILLIKER, personal communication). However, contrary evidence comes from the finding that replacement of *X*-heterochromatin with the *Y* chromosome did not reduce suppression in three $Su(bw^D)$s, even though the *Y*-bearing nucleolus is encoded on the opposite arm of the resulting constructs (S. HENIKOFF, unpublished results). Instead, we suggest that suppression is a property of *X* chromatin in general. This suggestion is motivated in part by the distribution of *Su(Nella)* second breakpoints, which are located in both medial and distal regions of chromosome *3* but only in distal regions of chromosome arm 2R. We suppose that relocation of bw^D to a position in 2R euchromatin is less suppressing than relocation to equivalent positions in 3L and 3R euchromatin. This might be the case if bw^D consisted largely of sequence repeats that are relatively abundant in chromosome *2* heterochromatin. As a result, it would be necessary to move bw^D more distally along chromosome *2* than along chromosome *3* to reduce its tendency to coalesce with homologous sequences. A more extreme suppressing effect of *X*-linkage would be expected because *X* heterochromatin might differ greatly in overall sequence structure from the bw^D element (LOHE *et al.* 1993). Although the origin of bw^D is unknown, physical mapping studies to determine its sequence structure are in progress.

Whereas only a single *X*-linked *Su(Nella)* was obtained, it was the strongest suppressor among the 38 two-break rearrangements (diamonds in Figure 5B). This suggests that *X*-linked *Su(Nella)*s were much less frequently generated than expected, relative to autosomal *Su(Nella)*s. To account for this frequency difference,

we suppose that the large targets for joining provided by the very long chromosome *2* arm and the medial regions of chromosome *3* in the current study outcompeted the *X* chromosome; in the previous study, only the *X* and the very distal tips of chromosome *3* provided targets for joining, leading to suppression of bw^D/+. Competition between target breaks might have occurred in the *Su(Nella)* X-ray screen but not in the ethylmethanesulfonate screen for $Su(bw^D)$/+s because X-rays frequently induce multiple breaks (ASHBURNER 1990).

A novel *trans*-sensing effect: *E(Byron)* and *Su(Nella)* rearrangements could be accounted for in terms of the distance of bw^D from heterochromatin without considering the effect of rearrangements on homologue pairing. Therefore, it was surprising that rearrangements involving the homologous 2R chromosome arm enhanced the *Byron* phenotype just as effectively as equivalent rearrangements involving the *Byron*-bearing 2R arm (Table 2). This *trans*-sensing effect, in which a gene on one chromosome senses the state of the homologous chromosome via somatic pairing, is not due to local disruption of pairing as seen for enhancement of transvection effects (LEWIS 1954; GELBART 1982; LEISERSON *et al.* 1994). Unlike transvection disruptions, $R(bw^-)$-mediated enhancement effects can occur with breaks that are distal to the affected locus and are not remedied in heterozygotes with similar breakpoint locations. These features of the new phenomenon suggest that $R(bw^-)$s enhance the *Byron* phenotype by facilitating the interaction between bw^D and heterochromatin, which lie near opposite ends of a chromosome arm. How this might occur in the context of nuclear organization is illustrated in Figure 8. In $Byron/bw^-$ heterozygotes, the distal location of bw^D will resist the tendency for bw^D to loop into the chromocenter. In $E(Byron)/bw^-$, looping increases because bw^D is closer to the chromocenter, with the bw^- homologue carried along. In $R(bw^-)/Byron$, the roles are reversed, with *Byron* carried along. This model can account for the fact that nearly all two-break $R(bw^-)$s were broken close to *brown* and in heterochromatin, whereas *E(Byron)*s were usually broken farther away from *brown* and frequently in proximal euchromatin (compare Figure 4, A and B). Because $R(bw^-)$s would work indirectly by dragging distal 2R close to heterochromatin, a smaller fraction of rearrangements should be effective than were found for *E(Byron)*s that directly move bw^D close to heterochromatin. The smaller target size accounts for the lower frequency with which $R(bw^-)$s were obtained relative to *E(Byron)*s (0.75% *vs.* 2%).

This proposed relationship between homologue pairing and chromocenter formation is reminiscent of GELBART'S hypothesis that the distribution of breakpoints that disrupt *dpp* transvection can be understood in terms of the chromocentral organization of the nucleus (GELBART 1982). Indeed, his distribution of breakpoints relative to *dpp* and the chromocenter closely resembles what we find for enhancers of *Byron*.

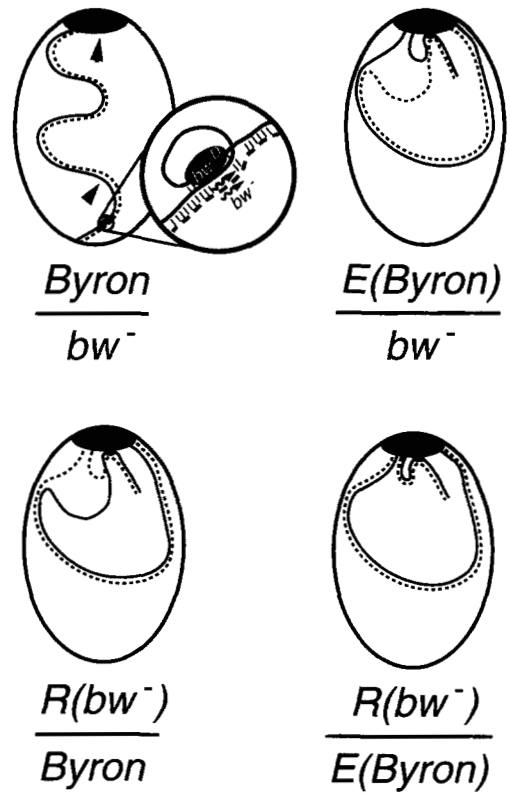

FIGURE 8.—Model for heterochromatin distance and *trans*-sensing effects on the *Byron* duplication (depicted in magnification). Four nuclei with different heterozygous combinations are shown with the chromocenter at the apical tip (large black oval), the chromosome carrying bw^D (solid line with small black oval), and its paired bw^- homologue (- - -). Arrowheads show the positions of breakpoints generating either *E(Byron)* or $R(bw^-)$ chromosomes.

Although the genetic phenomena are different, both probe the same underlying organizational features of the interphase nucleus, including paired homologues and a chromocenter consisting of fused heterochromatin. Whereas *dpp* heteroalleles are sensitive to their locally paired configuration, initiation of pairing is thought to begin far away at the chromocenter. Similarly, *para*-inactivation by bw^D is sensitive to its position relative to blocks of heterochromatin, however this position depends upon the configuration of its paired homologue. Although it might seem that homologue pairing and heterochromatin formation are distinct chromosomal processes, recent evidence (WAKIMOTO and HEARN 1990; DORER and HENIKOFF 1994) points to a shared mechanism in which the forces of somatic pairing underlie both (EPHRUSSI and SUTTON 1944). In this light, these forces are seen to be responsible for much of the structure of the nucleus, including both the spatial arrangement of euchromatic arms and the formation of heterochromatic chromocenters.

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