Heterochromatic Self-Association, a Determinant of Nuclear Organization, Does Not Require Sequence Homology in Drosophila

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ABSTRACT

Chromosomes of higher eukaryotes contain blocks of heterochromatin that can associate with each other in the interphase nucleus. A well-studied example of heterochromatic interaction is the *brown*^{Dominant} (*bw*^{D}) chromosome of *D. melanogaster*, which contains an ~1.6-Mbp insertion of AAGAG repeats near the distal tip of chromosome *2*. This insertion causes association of the tip with the centric heterochromatin of chromosome *2* (*2*h), which contains megabases of AAGAG repeats. Here we describe an example, other than *bw*^{D}, in which distally translocated heterochromatin associates with centric heterochromatin. Additionally, we show that when a translocation places *bw*^{D} on a different chromosome, *bw*^{D} tends to associate with the centric heterochromatin of this chromosome, even when the chromosome contains a small fraction of the sequence homology present elsewhere. To further test the importance of sequence homology in these interactions, we used interspecific mating to introgress the *bw*^{D} allele from *D. melanogaster* into *D. simulans*, which lacks the AAGAG on the autosomes. We find that *D. simulans bw*^{D} associates with *2*h, which lacks the AAGAG sequence, while it does not associate with the AAGAG containing *X* chromosome heterochromatin. Our results show that intranuclear association of separate heterochromatic blocks does not require that they contain the same sequence.

RECENT work has begun to uncover important interrelationships between the organization of the interphase nucleus and gene expression (reviewed in Francastel *et al.* 2000; Gasser 2001). One aspect of this organization is the positioning and interaction of heterochromatic regions of the chromosomes. Constitutive heterochromatin in most metazoan organisms makes up 5–50% of the genome. It consists of highly and moderately repetitive sequences, which are usually found flanking the centromeres. In interphase nuclei, heterochromatic sequences are often concentrated at the nuclear periphery and near the nucleoli. In a variety of cell types, centric regions cluster together but it is uncertain whether such clustering is sequence dependent (Cerda *et al.* 1999). For instance, the centric heterochromatin of different chromosomes often consists of the same or similar repeat sequences (Lee *et al.* 1997; Csink and Henikoff 1998). So, one may suppose that such clustering may be due to interactions among homologous sequences. However, these repeats also bind the same proteins, sometimes in a sequence-independent manner (Delattre *et al.* 2000; Saffery *et al.* 2000). Therefore it is unknown if the association of centric heterochromatin is due to heterochromatic protein-protein interaction, sequence similarity, arrangements dictated during mitosis and maintained during interphase, or another mechanism.

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In addition to the clustering of centric regions, another example of heterochromatic association was found with *bw*^{D}, an allele of the *brown* (*bw*) eye color gene of *Drosophila melanogaster*. The allele contains an insertion of ~1.6 Mb of heterochromatin into the *bw* coding sequence near the distal end of the right arm of the second chromosome (*2*R) at polytene band *59E* (Platero *et al.* 1998). Fluorescence *in situ* hybridization (FISH) of interphase diploid nuclei showed that in *bw*^{D} flies the distal tip of *2*R associates with *2*R centric heterochromatin (*2*Rh). Interestingly, in *bw*^{D}/*bw*$^{+}$ heterozygotes even the wild-type homolog associates with the centric heterochromatin (Csink and Henikoff 1996; Dernburg *et al.* 1996). This is because of the phenomenon of somatic pairing found in Dipteran insects. While homolog pairing is seen during prophase of meiosis I of all eukaryotes, it is seen only rarely in somatic nuclei. The exception is the Dipteran insects (Stevens 1908), where homologous regions of chromosomes are so close that, in interphase nuclei, *in situ* hybridization signals merge (Lifschytz and Hareven 1982; Fung *et al.* 1998). When *bw*$^{+}$ pairs with *bw*^{D}, both alleles associate with *2*Rh, which results in the partial silencing of the *bw*$^{+}$ gene on the homolog. It is hypothesized that positioning of the *bw*$^{+}$ allele near a large concentration of heterochromatin is responsible for its silencing (called *trans*-inactivation). The level of *trans*-inactivation of *bw*^{D}/*bw*$^{+}$ can be suppressed or enhanced by modifiers of position-effect variegation (PEV) as well as by chromosomal rearrangements that change the position of the *bw*^{D} insert relative to the centric heterochromatin. In these cases, the strength of silenc-

ing correlates with the level of association; that is, in the nuclei of flies where silencing is suppressed, we find a lower frequency of *bw*^{D}-*2*Rh associations (Talbert *et al.* 1994; Henikoff *et al.* 1995; Csink and Henikoff 1996).

As with the clustering of centromeric regions, the mechanisms of the association of *bw*^{D} and *2*Rh are not yet known. The heterochromatic insert in the *bw*^{D} allele consists mainly of the sequence repeat AAGAG. This satellite sequence is also found to some extent in the centric heterochromatin of all the chromosomes of *D. melanogaster*, but the pericentric heterochromatin of *2*R contains the largest concentrations of such a sequence (Lohe *et al.* 1993). The observation that *bw*^{D} can associate with *2*Rh may seem to imply an interaction of homologous sequences, perhaps analogous to somatic pairing of homologous chromosomes (Dernburg *et al.* 1996). This is a very attractive hypothesis, but a number of lines of evidence support the notion that *bw*^{D} is simply associating with the closest large block of heterochromatin to which it is linked. First, *bw*^{D}-*2*Rh association is partially suppressed by decreased dosage of HP1 (Csink and Henikoff 1996), a protein that binds to heterochromatin in a sequence-independent manner and, when mutant, is a classic suppressor of position-effect variegation (Eissenberg and Elgin 2000). Second, the somatic pairing of homologs is completed 30 min after mitosis and is prominent in embryos during and after embryonic cycle 14 (Hiraoka *et al.* 1993; Fung *et al.* 1998). However, *bw*^{D} associations are not set up until >5 hr into G_1 (Csink and Henikoff 1998) and are not seen in cycle 14 embryos (Dernburg *et al.* 1996). This suggests that the two types of chromosomal interactions are fundamentally different. Finally, transgene repeat arrays that form blocks of heterochromatin are sensitive to the distance of the block from centric heterochromatin (Dorer and Henikoff 1997), a property also seen with *bw*^{D} *trans*-inactivation (Talbert *et al.* 1994). Since the transgene array does not contain any apparent sequence homology to repeats of natural satellite DNA or other sequences present in pericentric heterochromatin, it was suggested that homology was not important for heterochromatic association (Dorer and Henikoff 1997). However, since the cytological association of the heterochromatic blocks in that study was not demonstrated, the conclusion that sequence was unimportant in heterochromatic association was tentative.

The association of *bw*^{D} with centric heterochromatin has been extensively cited as the archetype for an increasing number of instances where gene silencing has been correlated with association of a locus with heterochromatin in the space of the interphase nucleus (for review see Gasser 2001). Therefore, determination of the underlying mechanisms of heterochromatic associations is of key interest and will tell us if heterochromatin displays a general self-stickiness that is independent of the specific sequence content or if sequence-specific associations contribute to the tendency of chromosomal regions to colocalize. If sequence-specific interactions are not important, then molecules recognizing general features of large blocks of heterochromatin (such as repetitiveness, late replication, or specific histone modifications) may play a greater role. Conversely, if heterochromatic association is mechanistically related to somatic pairing, then the research on *bw*^{D} may not be applicable to systems other than Dipteran insects, because somatic pairing is not a phenomenon seen in most other organisms.

This article presents a series of experiments concerning heterochromatic associations in Drosophila diploid nuclei. First, we have examined heterochromatic associations in a homozygous-viable inversion chromosome with a block of heterochromatin in the middle of the chromosome arm. We show that heterochromatic associations analogous to those found with the *bw*^{D} chromosome are seen in chromosomes other than *bw*^{D}. Second, we address questions concerning the role of sequence similarity in heterochromatic association and find that such sequence similarity is unnecessary for heterochromatic association. We have done this by employing the ability of *D. melanogaster* to interbreed with a sibling species, *D. simulans*, which has divergent heterochromatic sequences. These results have important implications for our understanding of heterochromatic association, indicating that general properties of heterochromatin promote self-association in a sequence-independent manner. Additionally, our studies broaden our knowledge of how chromatin structure of specific chromosomal regions influences their placement in the interphase nucleus.

MATERIALS AND METHODS

Fly culture: All crosses were done at 25°. Flies were reared on standard yeast-cornmeal-molasses medium. Drosophila stocks were obtained from the Bloomington Stock Center, Andrew Davis, Daniel Barbash, or Steven Henikoff. The *T(2;4)E-1, T(2;3)5D,* and *In(2R)AT-4* rearrangements were generated in a previous study (Talbert *et al.* 1994; Henikoff *et al.* 1995). The *In(2R)AT-4* chromosome carries *bw*^{1}. The *T(2;4)E-1* chromosome contains a tandem duplication of the *bw* locus, one of which contains *bw*^{D} (called *Byron*). A *T(2:3)5D, bw*$^+$ chromosome was generated for this study by recombination of *T(2:3)5D, bw*^{D} with a wild-type chromosome. The wild-type *D. melanogaster* line used in all experiments described in this article was Canton-S.

Fluorescence *in situ* hybridization: FISH of diploid larval central nervous system (CNS) nuclei and the analysis of internuclear distances was performed as described previously (Csink *et al.* 2002 and further described below). The *59E* probe is a genomic clone in a P1 viral vector provided by the Drosophila Genome Project (P1 37-24; Smoller *et al.* 1991). The histone locus was probed with the plasmid phisBT provided by B. Wakimoto. The plasmid probe to the *Responder* repeat was provided by C.-I Wu. The 5S and 28S rDNA probes were generated by PCR according to the protocol in Talbert and Henikoff (2000) with the exception that *D. simulans v* DNA was used for the generation of the 28S probe. The AACAC (50 bp), AAGAG (50 bp), and dodecasatellite (DDS; 63 bp) (Carmena *et al.* 1993) probes were synthetic oligonucle-

otides. Nuclei were visualized using a Deltavision microscope (Applied Precision) with a cooled CCD camera and recorded at ×1000 original magnification. Each of the four wavelengths was corrected using the Deltavision three-dimensional deconvolution program (Applied Precision) and the stacks were projected into two dimensions for measurements. Using Softworx software (Applied Precision) we measured the two-dimensional distance between the closest signals of the two colors of interest and the area of the 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei. The radius of each nucleus was calculated on the basis of the area, assuming a circular shape of the nucleus. To ensure that measurements were unbiased by the experimenters' expectations, the image files were randomly coded and the codes were not broken until measurements were completed.

Differences in *D. melanogaster* and *D. simulans* heterochromatin were examined by FISH of mitotic chromosomes from third instar larval CNS cells. The starting lines of *D. melanogaster In(1)AB,w/FM6* and *D. simulans v (F6i-w)* were probed with the satellite sequences AACAGAACATGTTCG, AACAC, AAGAG, AATAATCATAG, AATAT, DDS, and AAGAC. Most of these probes had been previously examined in different lines of the two species by other workers. Our results for these specific lines conformed to the previously published distributions based on FISH of other lines from each species (Lohe and Brutlag 1987; Lohe *et al.* 1993; Carmena and Gonzalez 1995; Makunin *et al.* 1999). One exception is the AACAC probe, which had not been examined in *D. simulans.* Our results found that this probe did not hybridize to any sequences in *D. simulans* and confirmed that it hybridized to *2* and *Y* in the *D. melanogaster* starting line. AAGAG is found on all chromosomes in *D. melanogaster* and on the *X* and *Y* chromosomes in *D. simulans.* DDS is found only on *3* in *D. melanogaster* but is found mostly on *2* in *D. simulans* with a much smaller amount on chromosome *3.* The AAGAG, AACAC, and DDS probes were examined in the F_6 generation and used to show that the introgression of *D. simulans* heterochromatin was successful.

The 28S probe appeared as a "cloud" of staining within the interphase nucleus. To obtain a single point for measuring distances, we selected the AAGAG signal within the 28S "cloud" to use as the single point. This AAGAG signal would represent the AAGAG sequence located on the *X* chromosome, which was specified by the 28S probe.

For each data set (box plots in Figures 1 and 5) at least three wandering late third instar female larvae were examined with at least three separate fields per individual. Up to 10 randomly selected nuclei were analyzed from each field, for a total of at least 90 measurements for each genotype. When additional data were gathered, it was from additional larval slide preps, so that there were never >30 nuclei from a single larva in a data set. Each data set contains measurements from 90 to 177 nuclei. All nuclei in this study underwent the same experimental protocol.

Introgression: In our first attempt at interspecific introgression, we utilized *D. melanogaster In(1)AB, f^1* and *D. simulans C167.4* lines that we obtained from Andrew Davis. We crossed ~3500 *D. simulans C167.4* females and ~7000 *D. melanogaster In(1)AB, f^1*; bw^D*; st* males under the conditions described in Davis *et al.* (1996). Additionally, the male flies were collected when they were still virgins and aged for 10 days prior to mating with freshly eclosed virgin females (A. Davis, personal communication). From this cross we obtained only 77 F_1 females, all of which turned out to be infertile.

In our second attempt, we utilized *D. melanogaster In(1)AB,w/FM6* and *D. simulans v (F6i-w)* lines obtained from Daniel Barbash. We first crossed *In(1)AB,w/FM6* to a bw^D line and continued backcrossing to *In(1)AB,w/FM6* while selecting for bw^D for six more generations. We continued to backcross for six generations because it appears that there are mutations other than *In(1)AB,w* that are required for fertility rescue (Barbash and Ashburner 2003). After this preparation we crossed 100 *D. melanogaster In(1)AB,w/FM6 ;* bw^D/+ females and 100 *D. simulans v (F6i-w)* males (not virgin or aged) and obtained 293 F_1 flies. After backcrossing for six generations, we attempted to make homozygous stocks of each of the interspecific isofemale lines and discovered that the bw^D homozygotes appeared to be sterile.

RESULTS

Medial heterochromatin associates with centric heterochromatin: Although extensively cited as a model for the importance of nuclear organization in gene expression, the general applicability of the bw^D-*2*Rh association to other chromosomes that contain heterochromatin distally along the arm has been difficult to determine. Many chromosome rearrangements that move centric heterochromatin to the distal part of the chromosome arm are inversions that are viable only as heterozygotes. In flies, heterozygous inversions can form inversion loops via somatic pairing and cause all sequences near the break points to come closer to the chromocenter, hence mimicking association with centric heterochromatin. The association of the bw^D insertion is, so far, the only example where a distally located insertion of heterochromatin has been shown to associate with the centromere in a manner that could not involve the formation of an inversion loop. It is possible that the region around *bw* is unusual in its ability to associate with centric heterochromatin or that there is something unique about the bw^D heterochromatic insertion that promotes association.

To determine if other blocks of translocated heterochromatin could associate with the centric heterochromatin, we examined the inversion *In(2R)AT-4* using FISH of interphase nuclei from larval CNS cells as previously described (Csink and Henikoff 1996). *In(2R)AT-4* is homozygous viable, so no complications are introduced by inversion loops after homolog pairing. The breakpoints of this inversion were determined using polytene (Henikoff *et al.* 1995) and mitotic chromosome mapping and are diagrammed in Figure 1A, along with the three different probes used in this experiment. The proximal breakpoint in *In(2R)AT-4* moves the AACAC block of heterochromatin to a distal position so this probe can now be used to mark the position of distal *2*R. The position of AACAC can be compared to the position of the 5S rDNA repeat that is located in an analogous position on an unrearranged chromosome. To mark the position of the centric heterochromatin, the more proximally located *Responder* repeat was used. We compared the relative nuclear position of distal *2*R and *2*Rh in nuclei from either wild-type larvae or homozygous *In(2R)AT-4.* We found that the block of distally located heterochromatin in *In(2R)AT-4* does indeed promote the association of this region of the chromosome with regions surrounding the centromere (Figure

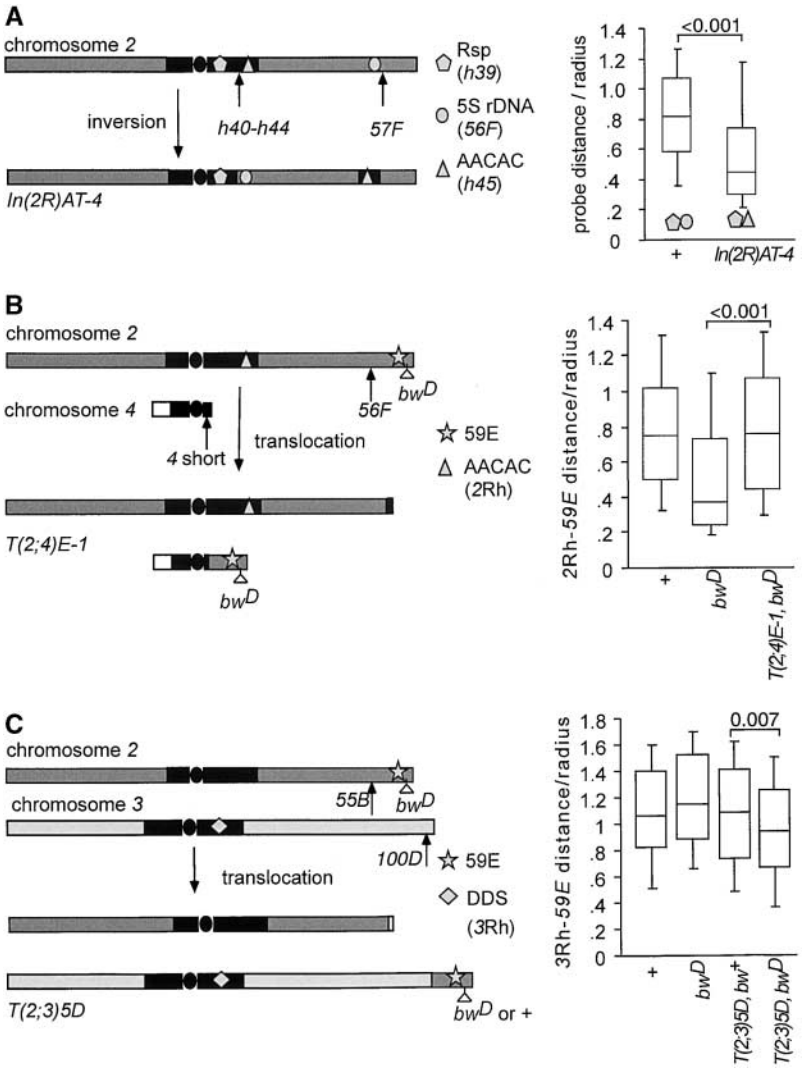

FIGURE 1.—Heterochromatic associations in nuclei containing chromosomal rearrangements. (Left) Diagrams of the rearrangement events in the various lines used. Euchromatic arms of the three chromosomes are shaded differently. Heterochromatin is solid. The solid circle represents the centromere. Arrows with numbers indicate the cytological locations of the breakpoints. (Right) Box plots showing the distributions of the distances between two probes corrected by the nuclear radius. Box plots are calibrated representations of histograms in which each horizontal line represents the tenth, twenty-fifth, fiftieth (median), seventy-fifth, and ninetieth percentiles. Each box plot presents measurements from at least 90 nuclei. *P*-values are indicated above the box plots and were determined using the two-tailed Mann-Whitney U-test for nonparametric comparison of two unpaired groups (SOKAL and ROHLF 1981). (A) Association of medially located heterochromatin in *In(2R)AT-4* with centric heterochromatin. The positions of the three probes in the inverted and normal chromosomes are indicated. The probes that were measured for each box plot are indicated by the shapes below the box. Rsp indicates the *Responder* heterochromatic repeat (WU *et al.* 1988). (B) In *T(2;4)E-1*, bw^D does not associate with *2*Rh. Chromosome *2* euchromatin is shaded and chromosome *4* euchromatin is open. The chromosomes are drawn to scale, showing that approximately one-quarter of the euchromatin from *2*R is translocated to *4*. Probe positions along the chromosomes are indicated. (C) In *T(2;3)5D*, bw^D associates with *3*Rh. Chromosome *2* euchromatin is darkly shaded and chromosome *3* euchromatin is lightly shaded. Probe positions along the chromosomes are indicated. DDS, dodecasatellite (CARMENA *et al.* 1993).

1A; compare box plots). Hence, we conclude that heterochromatic blocks other than those found in bw^D or located in the *59E* region can alter the organization of the interphase Drosophila nucleus.

Translocation of bw^D prevents association of sequences with the centric heterochromatin of *2*Rh: If bw^D heterochromatic associations result from sequence-specific interactions, then we might expect that a translocation of bw^D to another chromosome would still associate with *2*Rh, because *2*Rh contains the greatest concentration of the AAGAG satellite. Therefore, we examined the translocation *T(2;4)E-1* (HENIKOFF *et al.* 1995), which moves the distal region of *2*R (containing the bw^D heterochromatic insertion) onto chromosome *4* (Figure 1B). The phenotype of flies containing this translocation heterozygous with an unrearranged, wild-type chromosome (*T(2;4)E-1*, bw^D/+) is enhancement of bw^D *trans*-inactivation. This is in agreement with the heterochromatin distance effect described for such translocations (TALBERT *et al.* 1994; HENIKOFF *et al.* 1995). We examined the position of the *59E* and *2*Rh (star and triangle in Figure 1B) probes in the interphase nuclei of flies containing the homozygous translocations. No association of *59E* (now on the fourth centromere) with the centric heterochromatin of *2*Rh was apparent (Figure 1B). Although we could not mark the fourth chromosome and examine association directly, we speculate that bw^D most likely associates with the centric heterochromatin of chromosome *4*, which contains only 3% of the AAGAG satellite found on *2* (LOHE *et al.* 1993). An alternative explanation is that greater proximity to chromosome *4* enhances bw^D *trans*-inactivation without looping into contact with chromosome *4* heterochromatin. In either case, this experiment shows that the large amount of sequence homology on chromosome *2* does not dictate the nuclear position of the bw^D heterochromatic insertion.

We have also found evidence using a translocation of bw^D to the end of the third chromosome, *T(2;3)5D*, that bw^D will associate with the centric heterochromatin of *3*, which contains only 20% of the AAGAG found on *2* (LOHE *et al.* 1993; Figure 1C). This translocation results

in a phenotypic suppression of bw^D *trans*-inactivation. Previous work has found extensive correlations between the frequency of bw^D-*2*Rh associations and the strength of bw^D *trans*-inactivation (Talbert *et al.* 1994; Henikoff *et al.* 1995; Csink and Henikoff 1996). Therefore, as expected, the association with *3*h is not as prominent as that seen with an unrearranged bw^D chromosome and *2*h, although it is difficult to directly compare the two structurally different chromosomes (*2* and *3*).

These experiments show that the amount of sequence homology does not influence the choice of which centric block bw^D will associate with, and they support the hypothesis that sequence-specific interactions are not important for heterochromatic association and silencing.

Loss of AAGAG repeats in *2*Rh does not suppress the bw^D phenotype: If AAGAG sequences were required for bw^D heterochromatic associations and hence for the effect of bw^D on the phenotype, one would suspect that the deletion of AAGAG in *2*Rh would result in the suppression of *trans*-inactivation. We therefore examined the effect of the chromosome *Df(2R)M41A10*, which contains a deletion of most of the AAGAG sequence from *2*h (Lindsley and Zimm 1992; Figure 2A). We did not see such suppression. The amount of *trans*-inactivation in *Df(2R)M41A1,* bw^D/+ flies was the same as that in bw^D/+ flies (Figure 3A). While these data suggest that centric heterochromatin homology is unimportant for bw^D association, the above observation is not conclusive since not all of the AAGAG sequence is deleted from *2*h and the heterochromatic region of the wild-type homolog still contains AAGAG.

Heterochromatin devoid of AAGAG repeats still associates with bw^D: To more directly test if sequence similarity was necessary for heterochromatic association and silencing, we wanted to obtain a Drosophila line that contained the bw^D allele but did not contain AAGAG satellite sequences in the second chromosome pericentric heterochromatin. Such a second chromosome can be found in *D. simulans*. *D. simulans* and *D. melanogaster* are sibling species whose euchromatic sequence differs by only 1–4% (Powell 1997) and are perfectly homosequential for chromosome *2* euchromatin (Ashburner 1989). However, their heterochromatic sequences are greatly divergent (Lohe and Brutlag 1987). For instance, *D. melanogaster* has AAGAG satellite sequences within the pericentric heterochromatin of all of its chromosomes, but *D. simulans* has only AAGAG satellite sequence on its sex chromosomes. An opportunity to directly test the role of sequence similarity was offered by a newly described process for obtaining fertile hybrids of these two species with certain strains (Davis *et al.* 1996). We therefore introgressed the bw^D allele from *D. melanogaster* into a *D. simulans* background.

Initially, interspecific mating of *D. melanogaster* and *D. simulans* was described as producing sterile or inviable offspring (Lemeunier *et al.* 1986). However, recently, lines that are able to produce viable and fertile offspring have been found (Davis *et al.* 1996). We utilized two of these lines, *D. melanogaster In(1)AB,w/FM6* and *D. simulans v (F6i-w)* (referred to below simply as *v*), kindly provided by Daniel Barbash (Barbash *et al.* 2000). The scheme of the cross is diagrammed in Figure 4. We crossed 100 *D. melanogaster In(1)AB,w/FM6 ;* bw^D/+ females and 100 *D. simulans v* males and obtained 293 F_1 flies. Of these 293, 75 were *In(1)AB,w/v;* bw^D/+ females. Twenty-five vials were set up in which 3 F_1 females were mated to 5 *D. simulans v* males. A total of 82 F_2 females, of which 30 were bw^D, were obtained. Of 30 bw^D, 16 were *v* homozygotes. These were backcrossed to *D. simulans v en masse* for four more generations, while selecting for bw^D, to replace the majority of the *D. melanogaster* DNA with *D. simulans* (Figure 4). At generation 6 we established 20 isofemale lines for further examination. The various isofemale lines differed in their health and their ability to give rise to bw^D homozygotes. The healthiest line that gave rise to bw^D homozygotes was selected and expanded for further study. This introgression line is referred to below as *D. simulans* bw^D. However, it should be kept in mind that the region closely linked to the bw^D insertion and perhaps other euchromatic regions are likely to be derived from the *D. melanogaster* parent. Matings of homozygous males and females from each of the isofemale lines produced no progeny, so the lines are maintained by phenotypic selection of bw^D heterozygotes.

To test whether the effect of bw^D on the bw^+ homolog was phenotypically different in a *D. simulans* background compared to a *D. melanogaster* background, we examined fly eyes. Previous studies of *D. melanogaster*/*D. simulans* F_1 hybrids that carried bw^D on the *D. melanogaster* chromosome found that *trans*-inactivation by bw^D was moderately suppressed. This was interpreted as due to slight local disruptions in somatic pairing due to divergence of the sequences in the two species (Henikoff *et al.* 1993). We found similar results for the F_1 flies in our study (data not shown). The F_2 flies showed a high level of variation in the bw^D phenotype (Figure 3B), but this variation is quite a bit less by the F_6 generation (Figure 3, C and D). Since local pairing around the *bw* locus is likely to be similar in the F_2 and F_6 generations, we interpret this as due to different backgrounds segregating in the F_2 generation. *Trans*-inactivation in the interspecific bw^D heterozygotes, which were backcrossed for six generations, is still suppressed compared to *D. melanogaster* bw^D heterozygotes (Figure 3D). Because the heterochromatic insertion in bw^D is into the coding region (Platero *et al.* 1998), homozygous bw^D flies (both *D. melanogaster* and *D. simulans*) completely lack *brown* expression.

FISH was performed on 9 of the 20 isofemale lines derived from the F_6 generation to ensure the absence of AAGAG sequences in the centric heterochromatin of the autosomes (Figure 2B). As expected, these lines contained AAGAG sequence in the heterochromatin of the sex chromosomes and AAGAG sequence on the tip

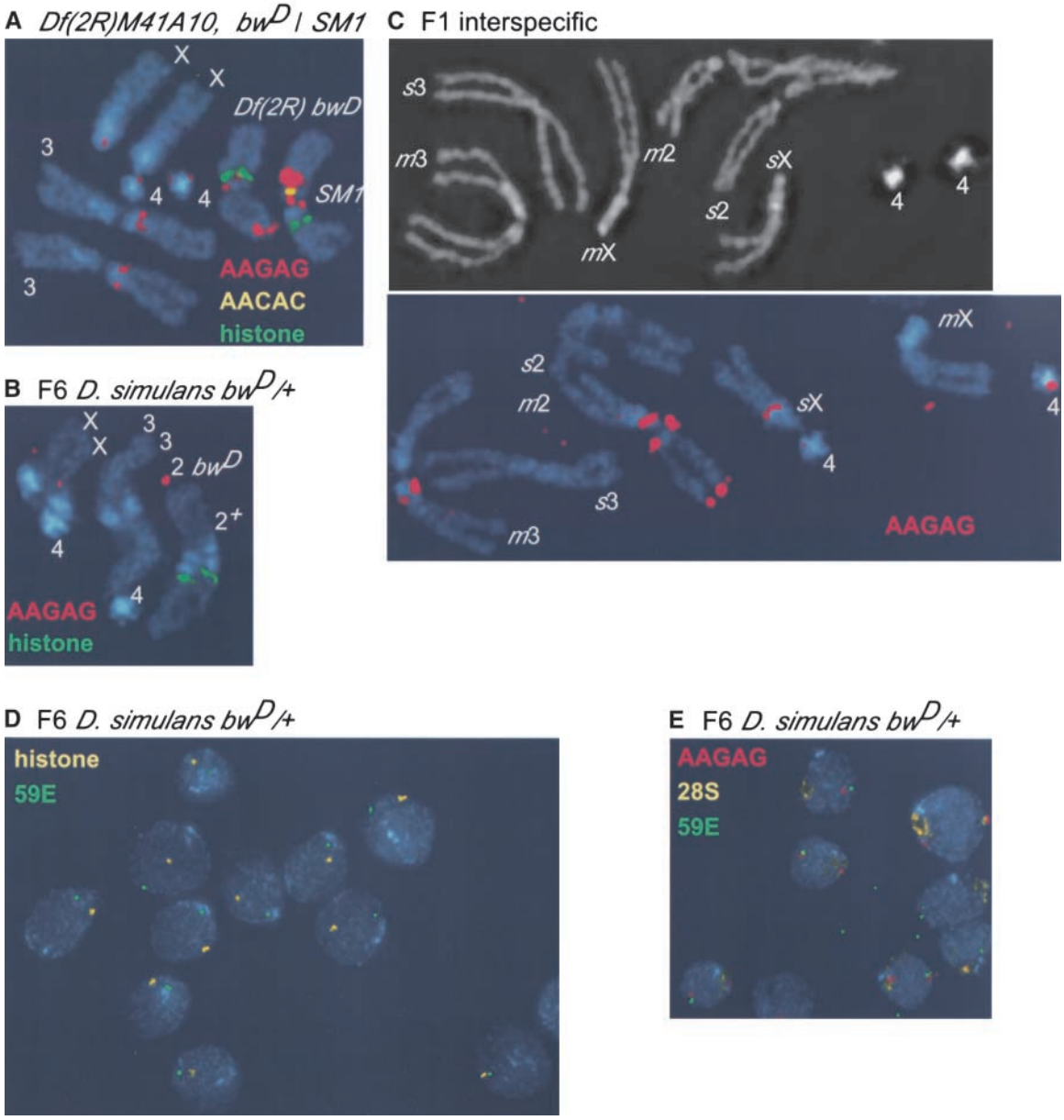

FIGURE 2.—Mitotic chromosomes and interphase nuclei. All chromosomes are from CNS cells of wandering third instar female larvae. Genotypes and probe colors are as indicated. All chromosomes are stained with DAPI (blue). (A) *Df(2R)m41A10, bw^D/* SM1. SM1 is a multiply rearranged balancer chromosome. (B) *D. simulans bw^D/+* from the F_6 generation of the introgression. The AAGAG signal on the *X* and the *bw^D* chromosomes do not overlap with the DAPI stain because this satellite sequence is not stained with the DAPI dye (PLATERO *et al.* 1998). (C) Mitotic chromosomes from F_1 interspecific hybrids. *s* indicates the chromosome from the *D. simulans* parent and *m* indicates the chromosome from the *D. melanogaster* parent. (Top) Chromosomes were not subjected to treatment for FISH to better show the detail of chromosome structure. (Bottom) Chromosomes hybridized with AAGAG. (D) Representative interphase nuclei from *D. simulans bw^D/+* used to measure the association between *2*h and *bw^D*. Data from such nuclei are displayed in the eighth box plot in Figure 5B. (E) Representative interphase nuclei from *D. simulans bw^D/+* used to measure the association between *X*h and *bw^D*. Data from such nuclei are displayed in the eleventh box plot in Figure 5B.

of *2*R in the *bw^D* locus, but not in the heterochromatin of the autosomes. Two other probes, AACAC and DDS, that differentiate between *D. melanogaster* and *D. simulans* heterochromatin (see MATERIALS AND METHODS) were also used and showed that the introgression of *D. simulans* heterochromatin was successful in all 9 lines examined. All heterochromatic sequences appeared to be derived from the *D. simulans* parents. Examination of the mitotic chromosome content of each line did not reveal any gross cytological variability. Additionally, the condensation of heterochromatic regions appeared normal in the mitotic squashes. However, the number of mitotic figures examined was few and performed on chromosomes treated for FISH, which is not ideal for

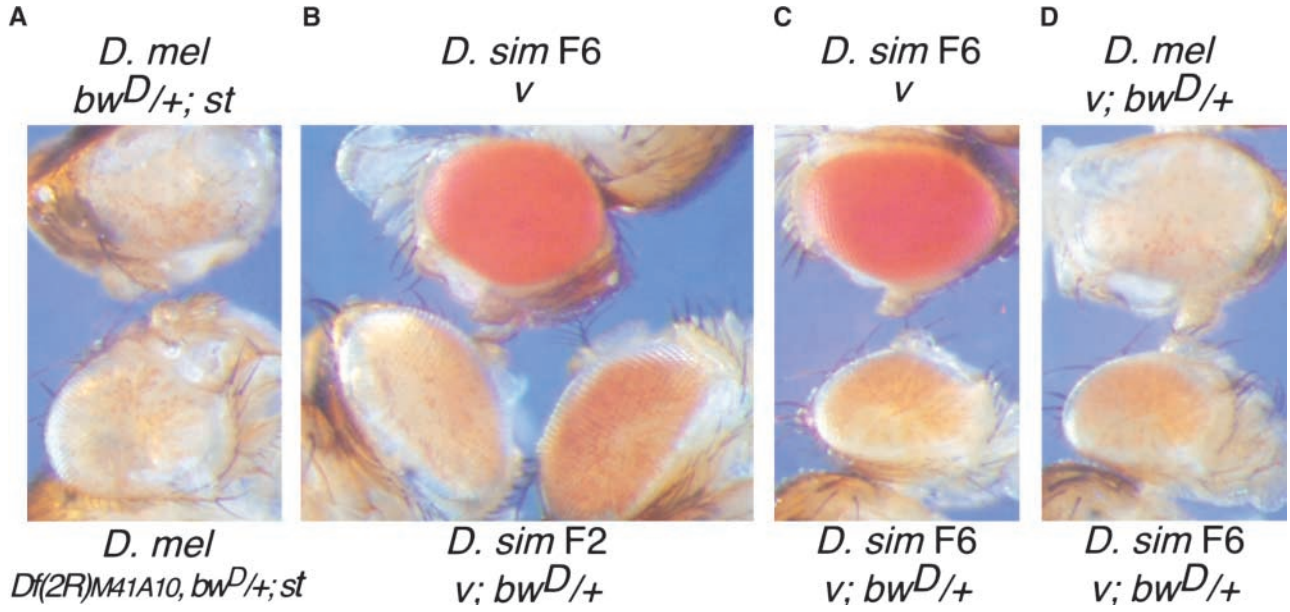

FIGURE 3.—*bw*^{D} *trans*-inactivation in various genetic backgrounds. Two pigments (brown and red) make up the dark red color of a wild-type Drosophila eye. The red pigment requires the *bw* gene and the brown pigments require the *st* and *v* genes. Therefore a fly will be white eyed if the *bw* gene is inactivated and the *st* or *v* genes are inactivated. Flies in the photos are male (aged 5–6 days). Genotypes are as indicated near each eye. (A) *D. melanogaster bw*^{D}/+; *st* compared to *Df(2R)m41A10 bw*^{D}/+ ; *st*. (B) *D. simulans v* compared to interspecific *v*; *bw*^{D}/+, which was backcrossed for two generations. (C) *D. simulans v* compared to interspecific *v*; *bw*^{D}/+, which was backcrossed for six generations. (D) *D. melanogaster yv*; *bw*^{D}/+ compared to interspecific *v*; *bw*^{D}/+, which was backcrossed for six generations.

detailed assessment of chromosome structure. Therefore, conclusions concerning the chromosomal architecture are limited and await further study.

We used FISH to test the association between *bw*^{D} and blocks of centric heterochromatin from different chromosomes in nuclei from third instar larval CNS cells. Previous studies measured the distance between a probe specific to the centric heterochromatin of *2*Rh and a single copy probe just proximal to *bw* at polytene band *59E*. Distances between probes were measured in 96–177 nuclei. When probe distances in nuclei from *bw*$^+$ and *bw*^{D} larvae were compared, the distance be-

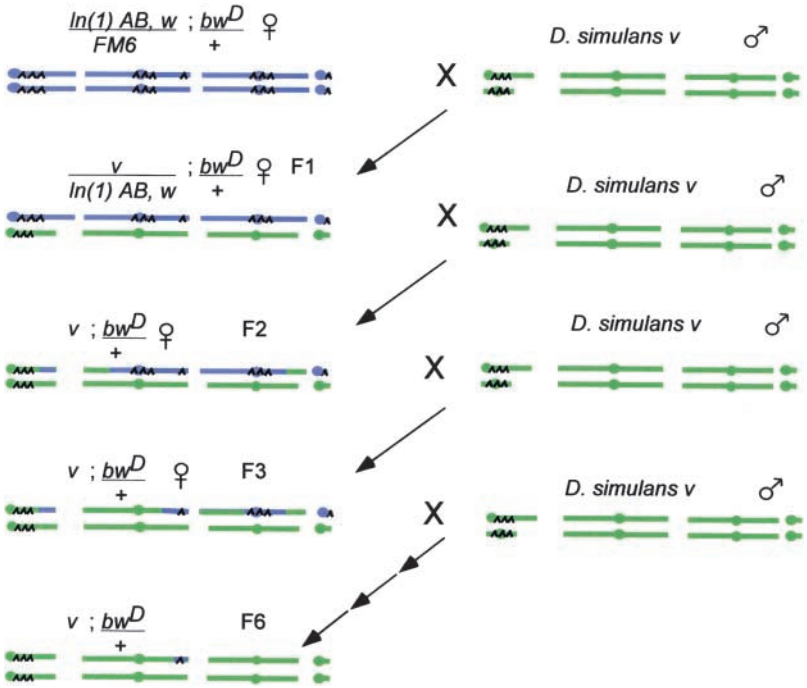

FIGURE 4.—Introgression scheme. Blue represents *D. melanogaster* chromosomes. Green represents *D. simulans* chromosomes. Chevrons represent locations of the AAGAG satellite sequence.

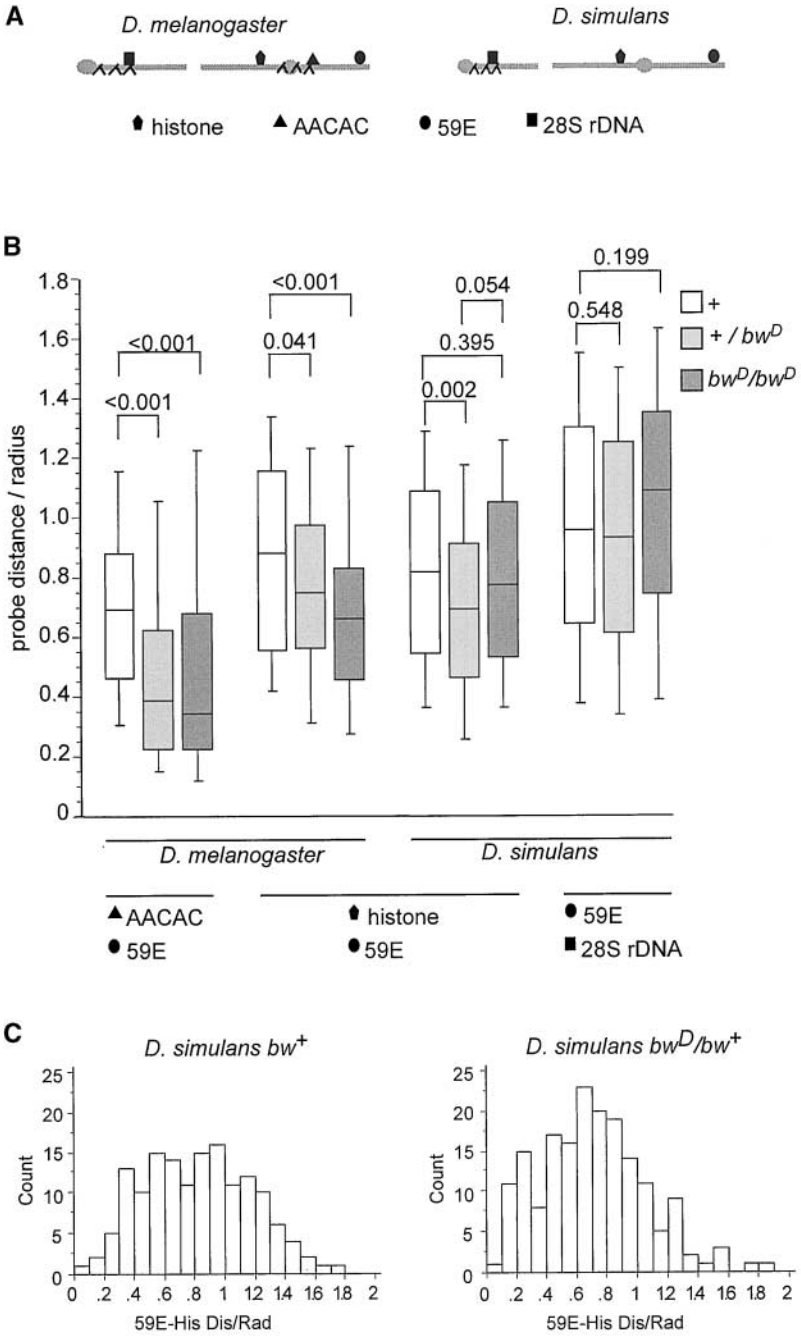

FIGURE 5.—Heterochromatic associations in the interphase nuclei of *D. melanogaster* and *D. simulans* larval CNS. (A) The location of the four different probes used in this study (indicated by the different shapes) and the concentrations of AAGAG repeats on the *X* (acrocentric) and *2* (metacentric) chromosomes. (B) Box plots showing the distance (corrected by the radius of the nucleus) between the *bw* locus probe (*59E*) and the *2*Rh probe (AACAC), the *2*Lh (His) probe, or the *X*h (28S) probe. Probes are indicated below and genotype is indicated by the shading of the box. Each box presents measurements from 96 to 177 nuclei. An explanation of box plots is provided in the legend of Figure 1. (C) Histograms of data from the seventh and eighth box plots in B.

tween the probes was significantly less in the bw^D nuclei, indicating that the heterochromatic insertion was causing the distal tip of *2*R to reside closer to *2*Rh in the space of the interphase nucleus. This previous study was repeated and results are shown in Figure 5B (first set of box plots) for comparison with the new results.

We examined the level of association between the *59E* region and either *2*h (which lacks AAGAG) or *X*h (which contains AAGAG) in interphase nuclei from the *D. simulans* bw^D line (Figure 2, D and E). Data in Figure 5B (fourth set of box plots) indicate that the *bw* region is not associating with the *X* chromosome heterochromatin even when the bw^D allele is present.

In the earlier study in *D. melanogaster*, a sequence (AACAC) was used that uniquely marked *2*Rh in females (CSINK and HENIKOFF 1996). This sequence is not present at all in *D. simulans* heterochromatin, so we used the histone repeat, which is located at the euchromatin-heterochromatin boundary on *2*L (Figure 5A). To ensure that we could still detect association using this probe, we examined the association of *59E* and the histone repeat in *D. melanogaster* in the same nuclei in which we reexamined the AACAC-*59E* association. Figure 5B (second set of box plots) shows that bw^D heterochromatic associations can be detected in *D. melanogaster* using this probe.

We then measured the *59E-histone* association in interphase nuclei from a *D. simulans* bw^D and a *D. simulans* bw^+ line derived from the introgression line, which segregates bw^D. In bw^D heterozygotes, *59E* associates with the second chromosome heterochromatin, which lacks AAGAG, at a significantly higher level than it does in bw^+ *D. simulans* (Figure 5B). Therefore, we see much the same association effects in bw^D/+ *D. simulans* as we see in bw^D/+ *D. melanogaster* (compare second and third sets of box plots in Figure 5B). This observation indicates that this heterochromatic association is not sequence dependent.

The above results from the *D. simulans* bw^D heterozygotes are quite similar to those seen in *D. melanogaster* heterozygotes. However, we also examined *D. simulans* bw^D homozygotes and obtained unanticipated results. In *D. melanogaster* lines there is the same, or a greater, level of association in the bw^D homozygotes as in the heterozygotes (Figure 5B, third set of box plots). However, in the *D. simulans* bw^D, we saw no significant difference between the bw^D homozygotes and bw^+ in the distance between *59E* and the histone repeat (Figure 5B, third set of box plots). As discussed below, we speculate that this is due to differences in the abundance of heterochromatin between the two species. Such a difference can be seen for the *X* and third chromosomes in the mitotic figures from interspecific F_1 hybrid females (Figure 2C).

DISCUSSION

Using various manipulations of chromosome structure and content, we have shown that heterochromatic blocks along a chromosome tend to associate with each other. Our experiments demonstrate that these heterochromatic associations between chromosomal regions in the interphase nucleus do not require that similar sequences be present in the different heterochromatic blocks. Therefore, heterochromatic association involves mechanisms distinct from those that result in somatic pairing. Since the tendency of the heterochromatin in bw^D to specifically associate with *2*Rh is not due to similar sequences in the two locations, it is probably due to the organization of the interphase nucleus into chromosomal domains. In higher eukaryotes, chromosomes do not range over the whole space of the interphase nucleus or mix freely with chromatin from other chromosomes. Instead, chromosomes are confined to a subspace of the interphase nucleus (for review see Parada and Misteli 2002). Our results imply that movement of chromosomal regions (in this case the distal region of the chromosomes arms) within their own domain may be easier than movement between domains. Thus the reason that bw^D associates with *2*Rh is because it is the largest block of heterochromatin within its own domain. The results presented above indicate that when bw^D is moved to another chromosome, it will associate with heterochromatic sequences within its new chromosomal domain.

It is likely that heterochromatic associations within a domain are mediated by proteins that recognize general features of heterochromatin such as repetitiveness, low transcriptional activity, late replication, or specific histone tail modifications found in heterochromatin, such as hypoacetylation or methylation at certain sites. Hence a general feature of heterochromatin may be self-stickiness that is independent of the specific heterochromatic sequence. This tendency of heterochromatin to self-associate may help to establish a higher-order structure in the interphase nucleus.

In these studies, we examined the role of heterochromatin in setting up the relative positioning of chromosomal regions within the interphase nucleus. Each of the examples we use involves the aberrant positioning of a constitutive heterochromatic block distally along the chromosome. While these chromosomal rearrangements may influence gene expression, this is not a situation expected to be found in a normal chromosome set. However, these aberrant chromosomes mimic a common normal situation. In higher organisms, as a cell differentiates and expression programs change, blocks of euchromatin become more condensed, creating facultative heterochromatin. Indeed, the increased condensation of chromatin is a feature of differentiated cells (Francastel *et al.* 2000). In normal nuclei, the creation of a region of facultative heterochromatin along an arm could promote the localization of that region with larger concentrations of heterochromatin in the nucleus. In this manner, euchromatic gene silencing could be initiated or enhanced by relocalization to a heterochromatic neighborhood (Brown *et al.* 1997; Francastel *et al.* 1999). This situation is analogous to the chromosomal rearrangements described here except that the heterochromatin is constitutively present in the rearrangements. Since this study has shown that heterochromatic associations are sequence independent, a change in chromatin structure that promotes association with constitutive heterochromatin could be acquired by other means. For instance, silencing may be accomplished by targeting proteins to a euchromatic region. These proteins may alter the chromatin structure to mimic constitutive heterochromatin and promote the tendency of that region to "stick" to other blocks of heterochromatin. Obvious candidates for these proteins would be those coded for by genes whose loss results in suppression of PEV, the suppressor of variegation [*Su(var)*] genes. PEV is the silencing of normally euchromatic genes when they are placed near or within heterochromatin. Indeed, *Su(var)* genes have been shown to influence nuclear organization as well as PEV (Csink and Henikoff 1996; Hari *et al.* 2001).

The method of interspecific introgression in Drosophila used in this study has not yet been widely used outside of studies on mechanisms of speciation (Bar-

BASH *et al.* 2000). A number of intriguing features of these experiments may address some fundamental questions concerning the divergence of heterochromatin in different species. For instance, we failed to see bw^D-2h association in homozygous bw^D *D. simulans* nuclei. This could be explained by the extensive quantitative and qualitative differences between the heterochromatin of the sibling species. LOHE and BRUTLAG (1987) examined 10 *D. melanogaster* simple satellite sequences in *D. simulans*. Of the 10, 7 could be identified in *D. simulans*; however, their abundance is much less in *D. simulans*. While these satellite sequences amount to 21% of the genome in *D. melanogaster*, they account for only 5% in *D. simulans*. The *X* and *Y* chromosomes in *D. simulans* are notably shorter than those in *D. melanogaster*, as a result of the differences in the heterochromatic regions (Figure 2C; LOHE and ROBERTS 1988). We have also found that the third chromosome appears to have less centric heterochromatin in *D. simulans* (Figure 2C). Since *D. simulans* has less total heterochromatin, the introgressed bw^D insertion will make up a greater proportion of the heterochromatic content of these nuclei. Therefore, an explanation for our results could be that the bw^D heterochromatic insert influences the overall distribution of heterochromatic proteins. An equivalent effect seen in *D. melanogaster*, the heterochromatin dosage effect, was originally described to account for the fact that an extra *Y* chromosome in *XXY* females suppresses PEV. Changes in *Y* chromosome dosage have been shown to modify the *trans*-inactivation phenotype of bw^D in *D. melanogaster* (CSINK *et al.* 2002). The functional basis of the heterochromatin dosage effect is believed to be a titration of a fixed amount of heterochromatic proteins by the addition of extra heterochromatin, so that the heterochromatin is less densely covered in heterochromatic proteins. Previous work has shown that some *Su(var)* mutations reduce the level of bw^D-2Rh associations (CSINK and HENIKOFF 1996), and suppression of PEV by second-site modifiers is thought to work through the same mechanism as the *Y* chromosome effect (*i.e.*, reducing the total amount of heterochromatic proteins binding the available heterochromatin) (SPOFFORD 1976; LOCKE *et al.* 1988). Therefore, we hypothesize that the addition of extra heterochromatin would suppress the association of bw^D and 2h. Consequently, two copies of bw^D may result in a heterochromatin dosage effect, thereby acting as a *Su(var)* and suppressing its own association with 2h in *D. simulans* bw^D.

An important requirement of the above model is the idea that somehow the total amount of heterochromatic proteins in *D. simulans* is actually lower than that in *D. melanogaster* to track the lower amount of heterochromatin. Otherwise, the ratio of heterochromatic proteins to heterochromatin in *D. simulans* would be greater than that in *D. melanogaster* and one would expect enhancement of the bw^D phenotype. Indeed, the segregation of genes coding for heterochromatic proteins from the two different species could contribute to the phenotypic variation seen in the F_2 generation (Figure 3B). Such changes in levels of chromatin proteins would be expected to have wide-ranging effects on the expression of many genes and global effects on chromatin structure. It is intriguing to think that one of the major differences between species may be the level of heterochromatic proteins. This suggests that further insight may be found by examining heterochromatin and heterochromatic protein variation in interspecific hybrids.

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