

Changes in Chromosomal Localization of Heterochromatin-binding Proteins during the Cell Cycle in *Drosophila*

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Abstract. We examined the heterochromatic binding of GAGA factor and proliferation disrupter (Prod) proteins during the cell cycle in *Drosophila melanogaster* and sibling species. GAGA factor binding to the *brown*^{Dominant} AG-rich satellite sequence insertion was seen at metaphase, however, no binding of GAGA factor to AG-rich sequences was observed at interphase in polytene or diploid nuclei. Comparable mitosis-specific binding was found for Prod protein to its target satellite in pericentric heterochromatin. At interphase, these proteins bind numerous dispersed sites in euchromatin,

indicating that they move from euchromatin to heterochromatin and back every cell cycle. The presence of Prod in heterochromatin for a longer portion of the cell cycle than GAGA factor suggests that they cycle between euchromatin and heterochromatin independently. We propose that movement of GAGA factor and Prod from high affinity sites in euchromatin occurs upon condensation of metaphase chromosomes. Upon decondensation, GAGA factor and Prod shift from low affinity sites within satellite DNA back to euchromatic sites as a self-assembly process.

FROM the time of their initial identification as “satellite” peaks of DNA on buoyant density gradients, long simple sequence repeats have presented an enigma. These sequences have no obvious function, and yet they are ubiquitous in higher eukaryotic genomes (John and Miklos, 1988). Repeat tracts can be greater than one megabase in length, and often comprise a large fraction of genomic DNA. In *Drosophila*, where the study of simple sequence DNA has been extensive, repeat arrays consisting of 5, 7, 10, and 12 mers account for half of all genomic DNA in some species, with extreme species-to-species variation that implies rapid generation and removal over evolutionary times (Lohe and Roberts, 1988). Nevertheless, within species, long, simple sequence arrays appear to be stable. Arrays typically reside in condensed heterochromatic regions of chromosomes in the vicinity of centromeres, suggesting a mitotic or meiotic function; however, the lack of conservation and the dispensability of most simple sequence arrays led to the view that simple sequence repeats are “junk DNA” (John and Miklos, 1988).

Proteins have been identified that bind to specific simple sequence arrays. In *D. melanogaster*, D1 protein binds to AT-rich simple sequences both in vivo (Alfageme et al., 1980) and in vitro (Levinger and Varshavsky, 1982). Evidence that AG-rich repeats are bound by the well-studied

chromatin-binding factor, GAGA, comes from examination of the staining pattern by anti-GAGA antibody on *D. melanogaster* metaphase chromosomes. Sites of binding correspond to N-banded heterochromatic regions (Raff et al., 1994), which are rich in (AAGAG)_n and (AAGA-GAG)_n arrays (Lohe et al., 1993). This is consistent with the inference that the extraordinarily high concentration of AG-rich sites leads to conspicuous binding, because the sequences that GAGA factor binds are AG rich. This prominent binding to heterochromatic regions in diploid chromosomes contrasts with the observation that no detectable GAGA factor binds to the heterochromatic chromocenter in polytene chromosomes (Raff et al., 1994; Granok et al., 1995), even though the protein is found at hundreds of euchromatic sites (Tsukiyama et al., 1994). This apparent discrepancy has been rationalized by noting that, because of selective amplification of euchromatin, polytene chromosomes are almost devoid of simple sequence repeats (Raff et al., 1994; Granok et al., 1995). Alternatively, it has been proposed that GAGA factor is actively excluded from the polytene chromocenter (Raff et al., 1994).

A third possibility to explain the difference between diploid metaphase chromosomes and polytene chromosomes in GAGA factor labeling patterns is that the protein binds to the AG-rich repeats only at mitosis. By this model, the protein shifts from euchromatic chromatin binding to mitotic binding of AG-rich repeats and back once per cell cycle. Because polytene chromosomes are perpetually in interphase, any AG-rich repeats present

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would not be expected to bind GAGA factor. Here we distinguish these models with the use of a heterochromatic insertion of AG-rich repeats, *brown*^{Dominant} (*bw*^D),¹ which we show has a substantial number of AG-rich repeats in polytene chromosomes. We confirm that N band-specific binding of GAGA factor is to the AG-rich repeat (AA-GAG)_n, and also demonstrate that this specificity is limited to mitotic chromosomes. Comparable results were obtained for proliferation disrupter (Prod) protein, a putative gene regulator thought to bind to a different satellite repeat (Torok et al., 1997).

Materials and Methods

Cytology

Orcein and DAPI (4,6-diamidino-2-phenylindole) staining were done as described by Ashburner (1990). Fluorescence in situ hybridization (FISH) oligonucleotide probes ([AAGAG]₇₋₁₀, [AACAC]₇₋₁₀, [AATAACATAG]₅, [GGTCCGACT]₄) were labeled according to Marshall et al. (1996). In situ hybridizations were performed according to Csink and Henikoff (1996) except that washes and hybridizations were done at 25°C for (AATAACATAG)₅. The 5' and 3' probes for *brown* were labeled by PCR using Dig-11-dUTP and Biotin-16-dUTP, respectively, where a 912-bp 5' product was amplified using 5'-GTAGGAGCCCAGGACGAA-CAA-3' and 5'-CCCCAGACTCATCATTAGACC-3', and a 1,019-bp 3' product using 5'-GCATTGAACTACATTTGAGC-3' and 5'-AAGGTGAGTAAGGGGTGGATA-3' primers. The 5' product corresponds to a site that is 5-kb upstream of the start of transcription of the *brown* gene, and the 3' product corresponds to a site that is 1-kb downstream of the polyadenylation (poly[A]-) addition site. The ratio of the hybridization level of the *bw*^D insertion to that in pericentric regions of a *bw*^D/*bw*^D diploid metaphase nucleus was calculated using the NIH Image (W. Rasband, National Institutes of Health, Bethesda, MD) analysis program.

For antibody detection we followed the method of Platero et al. (1995). Larval brains were incubated for 10 min in 0.5% Na citrate before formaldehyde fixation. The dilution of the primary antibodies was: 1:250 for the rabbit anti-heterochromatin protein 1 (HP1), 1:150 for rat anti-GAGA, and 1:5,000 for rabbit anti-Prod. Simultaneous detection of antibody and probe was done by successive application of FISH and antibody staining protocols preceded by a 2-min, 2% formaldehyde fixation step. For FISH (Csink and Henikoff, 1996), the acetylation and ribonuclease steps were omitted and pre- and post-hybridization washes were done at room temperature. After mounting and examination, coverslips were removed with acetone, and slides were immersed in TBST buffer of Platero et al. (1995) for antibody staining and detection after their procedure.

For bromodeoxyuridine (BrdU) labeling, wild-type third instar larvae were fed for 4.5 h with food containing 1 mg/ml of BrdU, and detected according to Ashburner (1990) using anti-BrdU-FITC (Boehringer Mannheim Corp., Indianapolis, IN). For aphidicolin uptake, early third instar larval brains were dissected in ringers solution and incubated at 22°C for 7 h in D22 *Drosophila* medium supplemented with 20 mM Hepes, pH 6.6 (Ashburner, 1990), in the presence or absence of 400 µg/ml aphidicolin (Calbiochem-Novabiochem Corp., La Jolla, CA). BrdU was added after 5 h of incubation. Brains were then incubated for 5 min in 0.5% sodium citrate, followed immediately by the antibody staining procedure.

Blot Hybridization Analysis

DNA extractions, Southern analyses, gel electrophoresis, and slot blots were done according to standard procedures (Ausubel et al., 1994). A PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) was used to quantify slot blot images to obtain ratios for each slot between hybridization of (AAGAG)_n and P1 genomic clone DS03480 (Berkeley *Drosophila* Genome Project, University of California, Berkeley, CA) used as probes (= hybridization ratio of Table I). Third instar larval salivary glands were

1. Abbreviations used in this paper: *bw*^D, *brown*^{Dominant}; PEV, position-effect variegation; Prod, Proliferation disrupter; DAPI, 4,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; HP1, heterochromatin protein 1; BrdU, bromodeoxyuridine; *Trl*, *Trithorax*-like.

used as polytene tissue and brains and imaginal discs as the source for diploid tissue.

Inverse and Direct PCR

To determine the exact insertion site and insert content of *bw*^D, an inverse PCR technique was used (Ochman et al., 1990) with the following modifications. Since there are no restriction sites for at least 20 kb within the insert, genomic DNA was cut with PvuII and the >20-kb fraction was gel purified. This DNA was then sonicated and the 2–2.5-kb fraction was gel purified and treated with Klenow DNA polymerase to make blunt ends. These fragments were circularized by ligation and amplified using divergent primers 5'-CAAGCCCGTGGTGGGTTTCATC-3' and 5'-CGGTGCGCGTGTGGCTATG-3' (5' nucleotides 5,322 and 6,587 in GenBank/EMBL/DBJ under accession number L23543), then re-amplified using nested primers 5'-CTCCTCGGCCAAGCTGAGTC-3' and 5'-GCCTCTCGAGTCGGATAAG-3' (5' nucleotides 5,271 and 6,624) from flanking *brown* sequence. Internal TaqI and AluI restriction sites were cleaved and fragments of 400–600 bp were gel purified and subcloned. Seven separate clones were sequenced. The flanking sequence, insertion site, and insert sequence (sequence data available from GenBank/EMBL/DBJ under accession number AF047460) agreed in all seven clones, although the sizes of the recovered inserts differed.

For the determination of the 3' junction, the PCR primer 5'-TCAATAGTAACCACTGCG-3', located 400 bp 3' from the junction, and the (AAGAG)_n oligonucleotide were used in a standard PCR reaction. The product was gel purified and sequenced.

Results

Mapping of the *bw*^D Heterochromatic Insertion

The mutation *bw*^D (*brown*^{Dominant}) was previously reported to contain large amounts of the simple sequence (AA-GAG)_n inserted near the distal tip of chromosome arm 2R (Csink and Henikoff, 1996; Dernburg et al., 1996). This provides a high concentration of a simple sequence removed from the bulk of heterochromatic repeats that could be exploited for investigation of the N band-specific binding of GAGA factor (Raff et al., 1994). To better ascertain the sequence structure of *bw*^D, first we precisely mapped the mutation. *bw*^D was originally reported to be an insertion (Hinton and Goodsmith, 1950; Slatk, 1955), and was later localized to within the coding region of the *brown* gene (Dreesen et al., 1988), which maps to the polytene band 59E1-2 or the interband just distal (Keizer et al., 1989). This mapping of the insertion to 59E1-2 is consistent with the appearance of a dense wedge-shaped region fused to 59E1-2 in *bw*^{D/+} heterozygotes (Fig. 1 A). The precise location of the insertion was determined by PCR amplification of both junctions. This revealed that the insertion is precise to within a few base pairs, and as a result, it interrupts Gly 577 of the *brown* gene open reading frame (Fig. 2).

It is possible that *bw*^D is not a simple insertion, but involves a more complicated rearrangement (Henikoff et al., 1993; Belyaeva et al., 1997). This was tested by FISH using flanking probes from the *brown* gene region. In polytene chromosomes stained with DAPI, the region on the distal side of 59E1-2 corresponding to *bw*^D fails to stain, as expected for AT-poor sequences (Manzini et al., 1983) (Fig. 1 B). This DAPI-dark region is closely flanked by the 5' probe on the distal side and by the 3' probe on the proximal side (Fig. 1 C). Because all of the extra DNA appears to lie between the two probes, we conclude that *bw*^D is a simple insertion.

Sequencing through both 5' and 3' junctions revealed

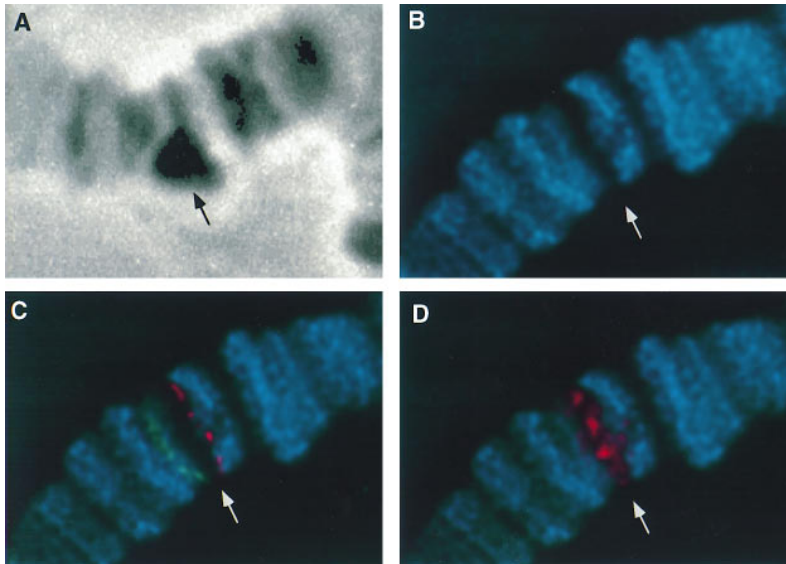


Figure 1. Cytological characterization of bw^D . (A) Orcein staining of third instar salivary gland polytene chromosomes from $bw^D/+$. (B) DAPI staining of bw^D/bw^D polytene chromosomes. (C) FISH of the same chromosome as in B, hybridized with probes to the 5' side (green) and to the 3' side (red) of the *brown* gene. (D) FISH with an $(AAGAG)_n$ probe (red) of the same chromosome as in B. Arrows point to the bw^D insertion.

that canonical $(AAGAG)_n$ repeats were predominantly present at both boundaries (Fig. 2). It was previously reported that there are few if any restriction sites extending inwards from either end of the insertion (Henikoff et al., 1993). We extended this restriction analysis of bw^D using *brown* gene-specific probes, digesting with a battery of 13 six-base and 8 four-base cutter endonucleases and failed to detect any sites for ~ 20 -kb inwards from the boundary (data not shown). This uninterrupted simple sequence appears to extend for 700 kb or more from both ends, because no sites were detected after digestion with EcoRI, EagI, and AvrII six-base cutter endonucleases, and electrophoresis on pulsed field gels (data not shown; Sabl, 1996). The existence of uninterrupted simple sequence DNA at both ends of the insertion argues against a transposon-mediated insertion event.

AAGAG Repeats Are Abundant in bw^D Relative to the Chromocenter of Polytene Nuclei

To determine the proportion of $(AAGAG)_n$ in bw^D relative to that in pericentric heterochromatin, we measured their amounts in diploid metaphase chromosomes of larval brains by comparing their relative hybridization intensities. These measurements indicated that the number of $(AAGAG)_n$ repeats in a single bw^D insertion is $17 \pm 2\%$ of that found in a haploid genome of wild-type females. Lohe et al. (1993) estimated the total amount of $(AAGAG)_n$ in females to be 8 Mb, so that the total amount of $(AAGAG)_n$ in bw^D is ~ 1.5 Mb, which is similar to the estimated size of simple sequence DNA obtained from pulsed

field gel analysis. We also examined the distribution of $(AAGAG)_n$ in bw^D polytene nuclei, where all of the pericentric and Y heterochromatin coalesces into a chromocenter. As expected from the extreme underrepresentation of simple sequence repeats in polytene nuclei, almost no labeling of the chromocenter was seen using an $(AAGAG)_n$ probe (Fig. 3, A and C–D). Surprisingly, we found prominent hybridization at the DAPI-dark region in bw^D (Figs. 1 D, and 3, A–B).

Two hypotheses can account for the labeling pattern of bw^D polytene nuclei: either the $(AAGAG)_n$ in the bw^D insertion is more fully replicated or its accessibility to probe is greater at bw^D than at the chromocenter. To distinguish these hypotheses, we measured amounts of $(AAGAG)_n$ from bw^D homozygotes and bw^+ by slot blot analysis in purified DNA of polytene and diploid tissues. We expected that the level of $(AAGAG)_n$ in bw^D diploid tissue would be 17% higher than in bw^+ (based on hybridization intensity of the bw^D insertion at metaphase). This minor difference would not be detectable by slot blot analysis, and the small average increase for bw^D seen in DNA from diploid tissue is not statistically significant (Table I). In contrast, the $(AAGAG)_n$ content of polytene salivary gland DNA from bw^D was significantly increased. The calculated amount of $(AAGAG)_n$ in the bw^D insertion is $\sim 100\%$ the level in wild type, several-fold more than the additional 17% measured in diploid metaphase chromosomes. Therefore, the heavier labeling at bw^D than at the chromocenter must be, at least in part, due to higher levels of replication of $(AAGAG)_n$ at the insertion relative to replication of this repeat at the chromocenter.

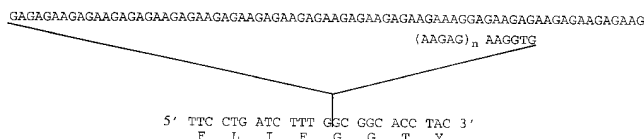


Figure 2. bw^D -*brown* junctions. The coding sequence around bw^D is depicted, corresponding to nucleotides 1,984–2,007 of Dreesen et al. (1988) and the insertion ends are shown above.

GAGA Factor Binds to $(AAGAG)_n$ -rich Repeats and Is Mitosis Specific

The ready detection of $(AAGAG)_n$ at the bw^D insertion makes it possible to test whether GAGA factor binds to this simple satellite sequence in polytene chromosomes. Immunolocalization shows that anti-GAGA antibody decorates numerous euchromatic bands in the vicinity of the *brown* locus, as expected (Tsukiyama et al., 1994), but fails

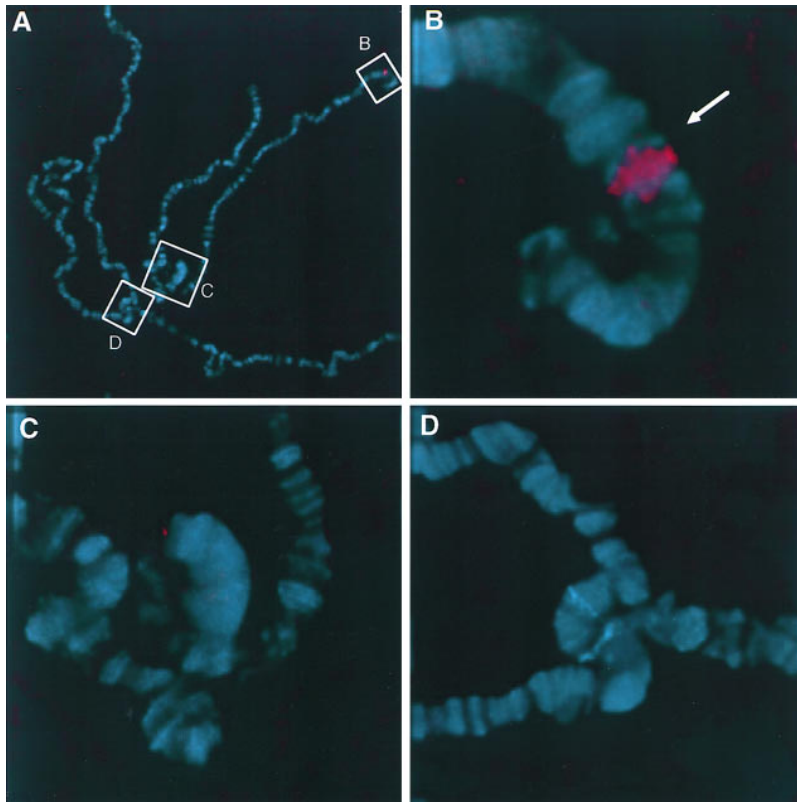


Figure 3. FISH with an (AAGAG)_n probe (red) hybridized to a polytene nucleus carrying *bw^D* stained with DAPI (blue). (A) The entire polytene nucleus. (B) Region of *bw^D*. Arrow points to the insertion. (C) Second and fourth chromosome heterochromatin. (D) X and third chromosome heterochromatin.

to decorate the DAPI-dark insertion (Fig. 4 A). This failure is not a consequence of a lack of accessibility to antibody, because in the same nucleus, anti-HP1 antibody prominently decorates the DAPI-dark insertion even more strongly than a previously reported site of HP1 localization at 60A nearby (James et al., 1989).

One possible explanation for the absence of GAGA factor at (AAGAG)_n of *bw^D* in polytene chromosomes is that (AAGAG)_n is a poor substrate for GAGA factor binding. To test this, we asked whether anti-GAGA antibody decorates the insertion in metaphase chromosomes. In embryos, GAGA factor was reported to be present most prominently in the pericentric regions of chromosome Y and 2, and at lower levels in X, 3, and 4 heterochromatin, consistent with binding to AG-rich repeats (Raff et al., 1994). Our results using larval brains confirm that these patterns are also found for this tissue (Fig. 5 A). In addition, larvae homozygous for *bw^D* also show GAGA factor binding at the 2R tips, the region of the *bw^D* insertion (Fig. 5 B). This indicates that absence of GAGA protein at *bw^D*

in polytene chromosomes is not because this insertion is a poor substrate for GAGA factor binding.

It appears that the substrate for GAGA factor binding to *bw^D* is (AAGAG)_n and not a different AG-rich repeat. The (AAGAGAG)_n satellite is repeated for the GAGA factor consensus sequence (Granok et al., 1995), but this simple sequence is absent from X and 3 heterochromatin (Lohe et al., 1993), sites that are decorated by anti-GAGA antibody (Fig. 5 A). Because (AAGAG)_n is the only known simple sequence array that both contains GAGA factor-binding sites and shows the observed pattern of binding, we conclude that GAGA factor binding is primarily to (AAGAG)_n-rich repeats in the *bw^D* insertion.

To confirm that (AAGAG)_n repeat blocks are substrates for GAGA factor binding, we compared the distribution of (AAGAG)_n (Lohe and Brutlag, 1987; Lohe and Roberts, 1988) (Table II) to GAGA factor binding (Fig. 5) in larval brains of sibling species. A perfect correlation was found. Both (AAGAG)_n hybridization and GAGA factor binding are confined to the X and Y chromosomes in *Drosophila simulans* (Fig. 5 C), and to the Y chromosome in *D. mauritiana* (Fig. 5 D). Therefore, sibling species that diverged from *D. melanogaster* only 2–3 million years ago (Powell, 1997) lack both AG-rich repeats and GAGA factor binding to mitotic chromosomes. A similar result was reported for *D. virilis* (Raff et al., 1994), which diverged from *D. melanogaster* 30–60 million years ago.

The presence of GAGA factor bound to (AAGAG)_n at *bw^D* in metaphase diploid chromosomes but its absence in interphase polytene chromosomes suggests that binding to (AAGAG)_n is confined to mitosis. Alternatively, there might be some special feature of salivary gland polytene chromosomes that excludes GAGA factor binding (Raff

Table I. Quantitation of Hybridization by (AAGAG)_n Probes

Nuclei	Genotype	Hybridization ratio	<i>bw^D/bw⁺</i> *
Diploid	+	10.8 ± 1.7	
Diploid	<i>bw^D</i>	12.5 ± 3.6	0.16
Polytene	+	1.3 ± 0.13	
Polytene	<i>bw^D</i>	2.6 ± 0.28	1.0

*Relative DNA content was calculated by subtracting the wild-type value from that for *bw^D* and normalizing to wild type. For diploid cells, the difference in hybridization levels is not significant ($P < 0.3$), and for polytene cells, the difference is highly significant ($P < 0.0001$).

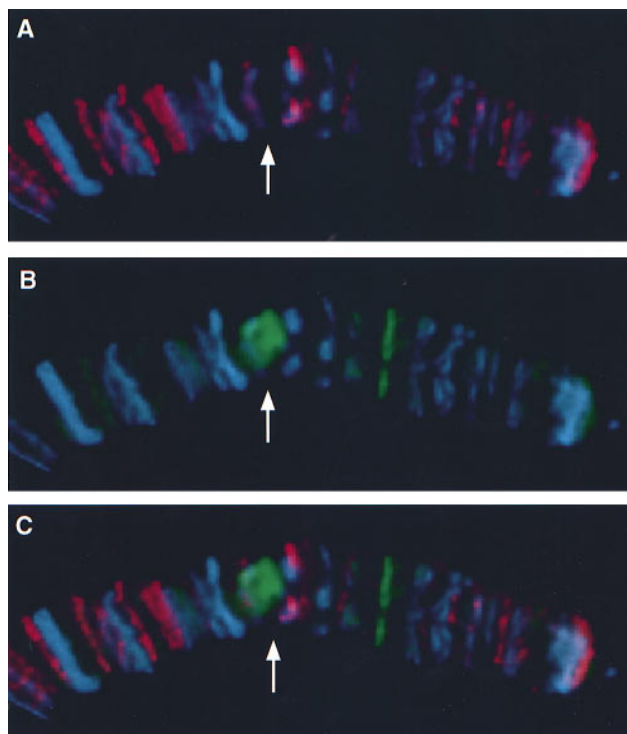


Figure 4. HP1 but not GAGA factor is present at *bw^D* in polytene chromosomes. (A) Tip of the 2R polytene chromosome carrying the *bw^D* insertion (arrow) stained with DAPI (blue) and labeled with anti-GAGA (red). (B) Same chromosome as in A, labeled with anti-HP1 (green). (C) Superimposition of A and B.

et al., 1994). To test this, we asked whether GAGA factor binding to $(AAGAG)_n$ occurs in diploid interphase chromosomes. In interphase nuclei from larval brains, anti-GAGA antibody predominantly shows a granular pattern, which we interpret as binding to dispersed euchromatic sites (Fig. 6 B). When these nuclei are probed with $(AAGAG)_n$, a clumpy pattern of labeling is seen (Fig. 6 D). Superimposition of the images reveals that the prominent clumps of $(AAGAG)_n$ labeling fall into holes of GAGA factor localization (Fig. 6, A and B, arrow). Lack of anti-GAGA binding in these holes is not a consequence of a lack of accessibility to antibody, because anti-HP1 appears to fill the holes (Fig. 6, E and F). We conclude that interphase polytene and diploid nuclei are similar in showing predominantly euchromatic GAGA factor binding.

Prod Binding Pattern Is Analogous to That of GAGA Factor

The unexpected absence of GAGA factor in the region of $(AAGAG)_n$ repeats in interphase nuclei prompted us to investigate the cell cycle binding specificity of Prod, which displays analogous polytene and metaphase localization patterns (Torok et al., 1997). Like GAGA factor, Prod labels ~400 euchromatic sites, but is excluded from the chromocenter in polytene chromosomes, and shows a restricted pattern of heterochromatin binding at metaphase. Torok et al. (1997) speculated that the sites of anti-Prod binding correspond to locations of $(AATAACATAG)_n$ satellite repeats in *D. melanogaster* (Lohe et al., 1993).

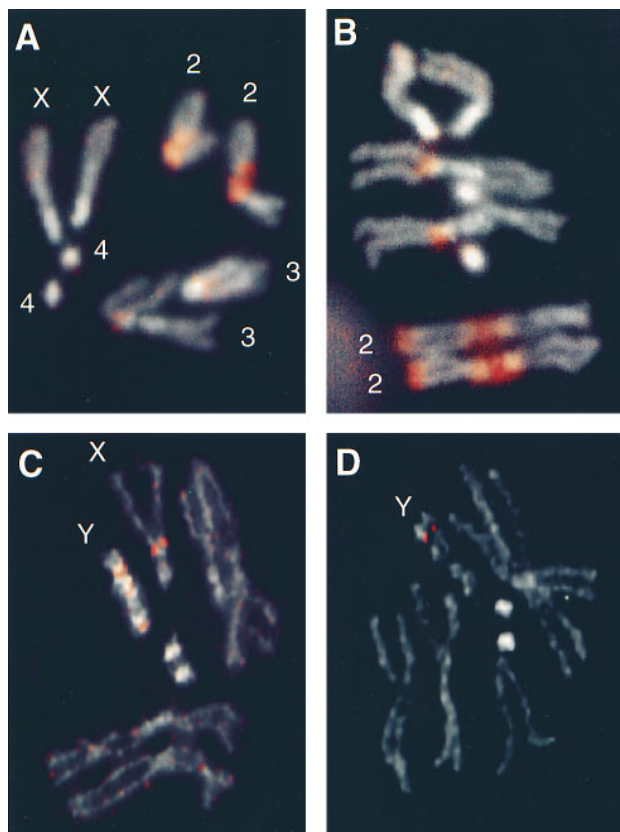


Figure 5. GAGA factor distribution on metaphase chromosomes of third instar larval brains of *bw^D* and sibling *Drosophila* species. (A) Chromosomes from wild-type *D. melanogaster*. (B) *D. melanogaster* chromosomes homozygous for the *bw^D* insertion. One tip of each second chromosome, at the bottom, is labeled. (C) Chromosomes from *D. simulans* males. (D) Chromosomes from *D. mauritiana* males. DAPI staining is shown in white and anti-GAGA labeling is shown in red.

Support for this hypothesis comes from examination of anti-Prod binding in brains of the closely related species *D. simulans* and *D. mauritiana*. *D. simulans* was shown to lack the $(AATAACATAG)_n$ satellite (Lohe and Brutlag, 1987), and we find that *D. mauritiana* also lacks this satellite (Table II). Consistent with the hypothesis that Prod binds to $(AATAACATAG)_n$, anti-Prod fails to label metaphase chromosomes of both *D. simulans* and *D. mauritiana* (Fig. 7).

Anti-Prod binding in larval brains is localized to chromosome 2 and 3 pericentric heterochromatin in metaphase chromosomes of *D. melanogaster* (Fig. 8 A), similar to

Table II. Chromosomal Localization of Satellite DNAs in *Drosophila melanogaster* and Sibling Species

	AATAACATAG	AAGAG	GGTCCCCTAGT	AACAC
<i>D. melanogaster</i>	2h, 3h	all h	3h	2h, Y
<i>D. simulans</i>	none	Xh, Y	2h, 3h	none
<i>D. mauritiana</i>	none	Y	2h, 3h	none

Xh, 2h, and 3h refer to X, second, and third chromosome pericentric heterochromatin, respectively. A dodecasatellite $(GGTCCCCTACT)_4$ probe (Carmena et al., 1993) provided a positive hybridization control, and an $(AACAC)_n$ probe (Marshall et al., 1996) provided a negative hybridization control for *D. simulans* and *D. mauritiana*.

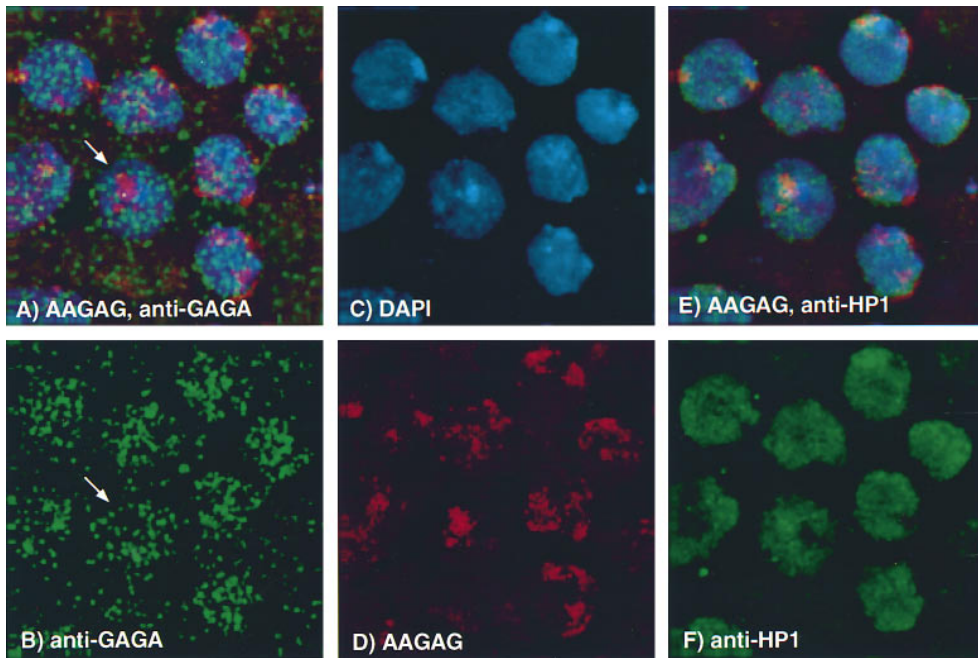


Figure 6. GAGA factor, HP1, and $(AAGAG)_n$ localization in interphase nuclei of third-instar larval brains. The different panels show the same field. (A) Composite showing nuclei stained with DAPI (blue), labeled with anti-GAGA (green), and hybridized with an $(AAGAG)_n$ probe (red). (B) Localization of anti-GAGA. (C) DAPI staining. (D) Localization of $(AAGAG)_n$. (E) Composite showing DAPI (blue), anti-HP1 (green) and $(AAGAG)_n$ (red). (F) Localization of anti-HP1. The near absence of anti-HP1 labeling of DAPI-bright regions (compare C to F) may indicate either that HP1 is sparse in AT-rich heterochromatin or that fixation caused preferential loss of HP1 from these arrays perhaps because of the ease of denaturation of AT-rich DNA.

what was reported for embryos (Torok et al., 1997). However, in the majority of interphase nuclei, a granular pattern is seen (Fig. 7, left panel; Table III), comparable to our results with GAGA factor. We interpret this granular pattern as predominantly euchromatic binding. This interpretation is supported by the pattern of anti-Prod staining to *D. simulans* and *D. mauritiana* brain interphase nuclei: these species, which lack $(AATAACATAG)_n$, display an exclusively granular pattern (Fig. 7).

Even though the majority of *D. melanogaster* interphase nuclei display a euchromatic anti-Prod pattern, 39% of nuclei display one or a few intense spots (Fig. 9; and Table III [total Prod spots/total nuclei = 39%]). Indeed, only 11% of nuclei bound by GAGA factor show intense spots (total GAGA spots/total nuclei = 11%). These intense GAGA spots occur in 90% of nuclei that also show intense Prod spots. The presence of intense spots in *D. melanogaster* interphases but their absence in sibling species that lack the corresponding satellites suggests that the spots represent interphase binding of GAGA factor and Prod to their satellite targets. Direct evidence that these intense spots occur

at satellite targets comes from examining *D. melanogaster* brain tissues labeled by FISH with $(AATAACATAG)_n$ and stained with anti-Prod. These probes colocalize in metaphase chromosomes and in interphase nuclei that display intense anti-Prod spots (Fig. 8), thus confirming the supposition of Torok et al. (1997) that Prod is targeted to $(AATAACATAG)_n$ -rich repeats.

Cell Cycle Dependence of Protein Binding to Satellite

The most likely explanation for the appearance of intense interphase spots is that they are either precursors or remnants of the satellite spots seen at mitosis. To determine if interphase spots are associated with mitotic events, larvae were fed the thymidine analogue BrdU before dissection of their brains, and the incorporation of BrdU was used to determine which nuclei had recently undergone S phase (Table III). Of the 26 interphase nuclei with intense spots of both GAGA factor and Prod, 23 (~90%) were labeled with BrdU, indicating recent passage through S phase. This compares with only 35% of nuclei overall that have

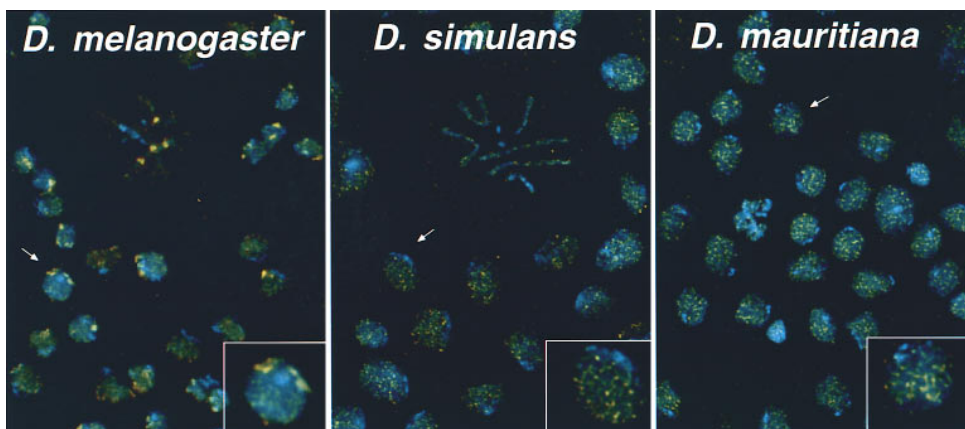


Figure 7. Prod localization in third-instar larval brain nuclei from *Drosophila* sibling species. DAPI staining is shown in blue and anti-Prod labeling in yellow. The arrows point to nuclei that are magnified at lower right.

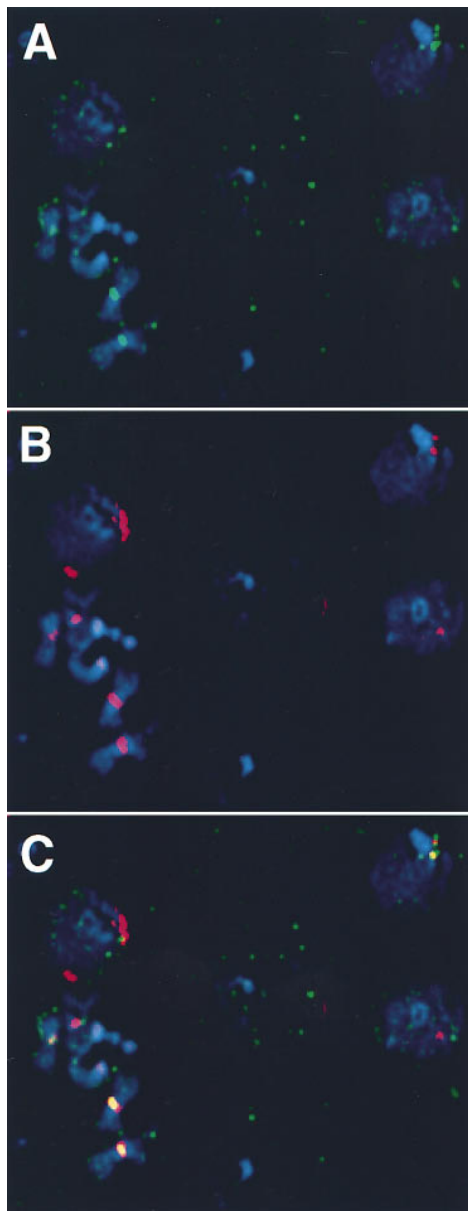


Figure 8. Colocalization of (AATAACATAG)_n and Prod. (A) Anti-Prod staining (green). (B) (AATAACATAG)_n FISH localization (red). (C) Composite. Blue indicates DAPI staining.

incorporated BrdU. Therefore, intense spots are seen exclusively in nuclei that have passed through S phase during the BrdU incorporation period, consistent with these spots appearing preferentially just before or just after mitosis. The fact that ~75% of the nuclei showing intensely labeled anti-Prod spots showed no intensely labeled anti-GAGA spots (Table III) suggests that heterochromatic Prod binding encompasses more of the cell cycle than heterochromatic GAGA factor binding.

We performed an experimental test of mitotic-specific protein binding to satellite by inhibiting progression through the cell cycle and asking whether this interfered with binding. Larval brains were incubated in the presence of BrdU and aphidicolin, a DNA polymerase inhibitor (Schubiger and Edgar, 1994), and examined for anti-Prod

Table III. Protein Localization and BrdU Incorporation

BrdU	GAGA granular		GAGA spots		Total
	Prod granular	Prod spots	Prod granular	Prod spots	
Unlabeled	125	42	0	3	170
Labeled	34	34	2	23	93
Total	159	76	2	26	263

Numbers of nuclei counted are shown.

and anti-BrdU staining. With anti-Prod, an exclusively granular pattern without intense spots was seen (Fig. 10 C); in contrast, control brains incubated without the inhibitor showed both a granular pattern and intense spots (Fig. 10 A). The inhibitor effectively prevented progression through the cell cycle because no anti-BrdU staining was seen (Fig. 10 D), whereas control brains showed anti-BrdU staining (Fig. 10 B). Therefore, the appearance of intense Prod spots requires progression through the cell cycle.

Discussion

Cytological Appearance of *bw^D*

Our characterization of the *bw^D* insertion in salivary gland chromosomes has identified it as orcein staining and DAPI-dark chromatin fused to the 59E1-2 band. In a recent cytological study of *bw^D*, the insertion was seen to lie just distal of the 59E1-2 band under conditions that reduce heterochromatin condensation (Belyaeva et al., 1997). The *brown* gene had been previously mapped to the distal edge of the 59E1-2 band or the adjacent interband (Keizer et al., 1989). These cytological observations are consistent with our FISH mapping and sequencing of the junctions, which suggest that *bw^D* is a simple insertion into the *brown* coding region.

The *bw^D* insertion displays heterochromatic properties in polytene chromosomes: in the Belyaeva et al. (1997) study, *bw^D* was found to retain orcein staining characteristic of α -heterochromatin and to bind anti-HP1 (Fig. 4 B). They inferred that the size of the *bw^D* insertion in polytene chromosomes is small relative to its size in diploid metaphase chromosomes and suggested that this is because of an underrepresentation in polytene chromosomes. This suggestion is consistent with our molecular mapping and cytological quantitation, which leads to an expected size of ~1–2 Mb, or on the order of a Bridges division on the polytene map. Among models for underrepresentation, the frozen fork model predicts a gradient of decreasing DNA density due to the stalling of polymerases transiting into simple sequence repeats (Laird et al., 1974). This is inconsistent with the lack of any constriction at the site of *bw^D*, evident from both Belyaeva et al. (1997) and the present work. No gradient is predicted by excision (Spradling, 1993) or copy-choice replication (Henikoff, 1996) models for underrepresentation. This cytological lack of a DNA density gradient agrees with previous molecular evidence that no frozen forks are present between fully replicated and underrepresented regions of polytene chromosomes (Glaser et al., 1992).

Despite apparent polytene underrepresentation at *bw^D*, the level of (AAGAG)_n is several-fold higher than at pericentric regions. A possible explanation for this differential

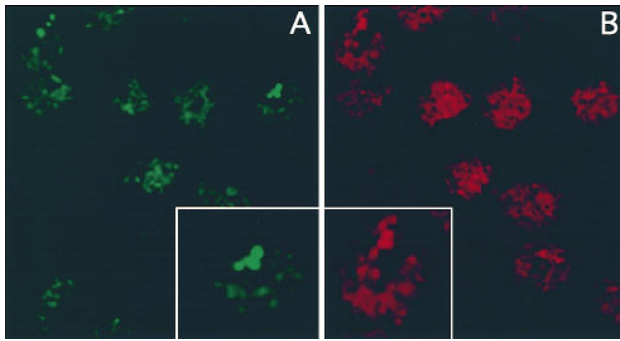


Figure 9. Prod and GAGA factor distribution in interphase nuclei. The same field is shown in each panel. (A) Anti-Prod staining (green). (B) Anti-GAGA staining (red). One nucleus from each field is magnified below.

behavior is that replication origins in flanking euchromatin are closer to $(AAGAG)_n$ in the bw^D insertion than they would be when $(AAGAG)_n$ is sandwiched between other blocks of simple sequence DNA in pericentric heterochromatin. Another possibility is that polytenization of euchromatic arms retards association between bw^D and pericentric heterochromatin along the chromosome. This possibility is consistent with the higher frequency of association between bw^D and 2R heterochromatin in diploid nuclei (Csink and Henikoff, 1996; Dernburg et al., 1996) than between bw^D and the chromocenter in polytene nuclei (Talbert et al., 1994; Belyaeva et al., 1997). Displacement of bw^D from the heterochromatic compartment would then allow higher euchromatic replication levels.

Together with the evidence that bw^D consists of $(AAGAG)_n$, the binding of anti-HP1 to bw^D in polytene chromosomes demonstrates that HP1 associates with heterochromatin consisting of simple sequence repeats. The

presence of middle repetitive sequences at the chromocenter and chromosome 4 has previously been shown to correspond to the location of anti-HP1 antibody binding (Miklos and Cotsell, 1990). Therefore, HP1 has a broad range of repetitive sequence chromatin substrates.

Is There a Mitotic Requirement for GAGA Factor and Prod Binding to Simple Sequence DNA?

Mutations in both *Trihorax-like* (*Trl*), which encodes GAGA factor, and *prod* cause chromosome condensation defects during cell division, indicating that these genes have essential roles in chromosome packaging (Bhat et al., 1996; Torok et al., 1997). In the case of *prod* mutations, the defects are limited to chromosomal regions where $(AATAACATAG)_n$ repeats are located (Torok et al., 1997). These observations suggest that binding of GAGA factor and Prod to their target satellite sequences are essential for normal mitosis. However, not all chromosomes carry these satellites or show binding of GAGA factor or Prod. Moreover, the sibling species *D. simulans* and *D. mauritiana* lack detectable $(AATAACATAG)_n$ entirely from the genome and lack detectable $(AAGAG)_n$ satellites on their autosomes. Because $(AAGAG)_n$ in *D. mauritiana* is found only on the Y chromosome, females have no detectable $(AAGAG)_n$. We have shown that binding of GAGA factor and Prod to heterochromatin in mitotic chromosomes of *D. melanogaster*, *D. simulans*, and *D. mauritiana* corresponds precisely to the location of their target satellite sequences. Therefore, we propose that these proteins are nonessential for chromosome condensation if their target satellites are not present. Conversely, the essentiality of GAGA factor and Prod binding to their target satellites in *D. melanogaster* would reflect a function that has been acquired during expansion of the satellite in the *D. melanogaster* lineage.

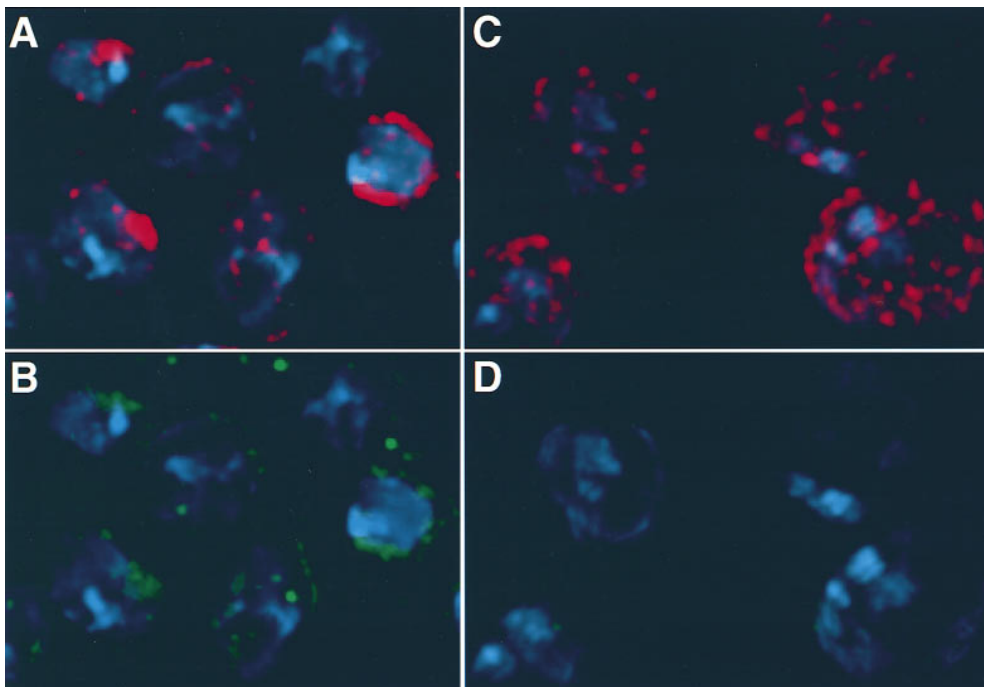


Figure 10. Anti-Prod and anti-BrdU staining of interphase nuclei in the absence (A and B) or presence (C and D) of the DNA inhibitor, aphidicolin. (A and C) Anti-Prod staining. (B and D) Anti-BrdU staining. A concentration of nuclei with intense Prod spots is shown in A, and the same field is shown in B, where BrdU uptake is seen for most nuclei. Only a granular anti-Prod pattern is seen in C for aphidicolin-inhibited nuclei that show no BrdU uptake (D), even when anti-Prod staining is emphasized as in this example.

Dosage-dependent Enhancement of Position-Effect Variegation by *Trl* Mutations

The localization of GAGA factor binding to sites in heterochromatin (Raff et al., 1994) was unexpected given that *Trl* mutations are dosage-dependent enhancers of position-effect variegation (PEV), whereas genes for heterochromatic-binding proteins like HP1 are dosage-dependent suppressors of PEV. This finding led to intricate models for enhancement of PEV based on heterochromatic binding of GAGA factor (Granok et al., 1995; Laible et al., 1997). However, the lack of detectable dosage-dependent modification by *Trl* mutations on *bw^D*-induced PEV argues against these models (Sass and Henikoff, 1998). Our finding that GAGA factor binding to heterochromatin is specific for mitosis provides an explanation for lack of modification, because PEV silencing occurs at interphase when there appears to be no role for GAGA factor in heterochromatin. Also, the lack of detectable GAGA factor binding in *D. mauritiana* females argues against any fundamental role for this protein in heterochromatin assembly. Rather, these dosage effects of *Trl* mutations probably result from GAGA factor's chromatin activation role (Paro and Harte, 1996), which would hinder formation of a silenced complex at PEV-affected reporter genes.

Cell Cycle-dependent Binding of GAGA Factor and Prod

Although the release of DNA-binding proteins at mitosis has been documented (Martinez-Balbas et al., 1995), their movement from one class of sites to another and back every cell cycle is a novel observation. Cell cycle-specific binding to sequences in heterochromatin is especially surprising given the original definition of heterochromatin as chromosomal material that remains condensed throughout the cell cycle (Heitz, 1928). Such cycling was not observed by Raff et al. (1994) and Torok et al. (1997), who observed pericentric binding of GAGA factor and Prod, respectively, throughout the cell cycle in early embryos. However, early embryonic nuclei cycle every 20 min, whereas cell cycles in larval brains last several hours (Ashburner, 1990), and there might not be enough time for these proteins to relocate from satellites to dispersed binding sites. Indeed, intense spots appear over a substantial portion of the larval brain cell cycle, suggesting a lengthy period during which GAGA factor and Prod move between their target satellite sequences in heterochromatin and dispersed sites in euchromatin. Consistent with this interpretation, a granular pattern of GAGA factor binding without the appearance of intense spots has also been shown for interphase tissue culture cells (Kellum et al., 1995).

The higher proportion of interphase nuclei with intense Prod spots (39%) to nuclei with intense GAGA spots (11%) indicates that Prod is bound to (AATAACA-TAG)_n for a longer portion of the cell cycle than GAGA factor is bound to (AAGAG)_n. This presents a difficulty in trying to explain how these proteins cycle. An active process might be involved in removing these proteins from their euchromatic sites at mitosis (Martinez-Balbas et al., 1995), and a different active process, such as the binding of HP1, might remove these proteins from their satellite sites at interphase. However, in this case, we would expect both

GAGA factor and Prod to come on and off their target satellites at the same time, which is not what we observe.

We propose that the binding and release of simple sequence satellites by their specific binding proteins can occur by a self-assembly mechanism. Dispersed high affinity binding sites in euchromatin would bind tightly, whereas satellite sequences in heterochromatin would bind with low affinity. In support of this possibility, we note that (AAGAG)_n differs from the consensus GAGA factor-binding sequence, GAGAGAG (Granok et al., 1995), and would be expected to bind GAGA factor less tightly (Omichinski et al., 1997). Mitotic chromosome condensation or some other active process would push off the proteins from their high affinity dispersed sites, making these proteins available for packaging the tandemly repeated sequences, whose biased composition might hinder normal condensation processes. Decondensation of dispersed high affinity sites after mitosis would make these available for binding once again, and so at equilibrium, the final distribution would depend upon binding site affinities and total numbers of factor molecules. By this scenario, the transit time for each protein could differ depending upon the details of the different protein-DNA interactions.

Cell cycle-dependent binding of GAGA factor, Prod, and presumably other DNA-binding proteins to their target satellites has general implications for understanding functions that satellite arrays may have acquired during evolution. Together with the mapping of centromeres to satellite-rich regions of higher eukaryotic genomes, our results are consistent with a role for satellite-binding proteins in chromosome segregation. Although no single satellite repeat has been shown to perform a cellular function in *Drosophila*, the particular sequence of a repeat unit might be unimportant. If different satellite arrays are redundant with one another, a high concentration of arrays in pericentric regions might be necessary for proper mitotic behavior.

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