In Vivo Chromatin Accessibility Correlates With Gene Silencing in Drosophila

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ABSTRACT

Gene silencing by heterochromatin is a well-known phenomenon that, in Drosophila, is called position effect variegation (PEV). The long-held hypothesis that this gene silencing is associated with an altered chromatin structure received direct support only recently. Another gene-silencing phenomenon in Drosophila, although similar in its phenotype of variegation, has been shown to be associated with euchromatic sequences and is dependent on developmental regulators of the *Polycomb* group (*Pc*-G) of gene products. One model proposes that the *Pc*-G products may cause a local heterochromatinization that maintains a repressed state of transcription of their target genes. Here, we test these models by measuring the accessibility of *white* or *miniwhite* sequences, in different contexts, to the *Escherichia coli dam* DNA methyltransferase *in vivo*. We present evidence that PEV and *Pc*-G-mediated repression mechanisms, although based on different protein factors, may indeed involve similar higher-order chromatin structure.

WHEN the euchromatic gene *white*, which is responsible for the red color of the fruit fly eye, is relocated near heterochromatin because of an *X*-chromosome rearrangement, *white* function is extinguished in some cells, leading to a mottled pigmentation. This phenomenon is called position-effect variegation (PEV). To explain the silencing of the *white* gene in some cells and not in others, the most popular model proposes that the condensed and inactive conformation of pericentric heterochromatin spreads over the breakpoint of the rearrangement and randomly inactivates neighboring genes (Locke *et al.* 1988; Eissenberg 1989; Tartof *et al.* 1989; Henikoff 1990). About 150 genes have been identified by genetic modifier mutations, both suppressors and enhancers, of this phenomenon (Reuter and Spierer 1992; Henikoff 1996). It has been proposed that the products of many of these genes encode chromatin components or modifiers of those components. Some suppressor mutations were indeed molecularly identified as heterochromatin constituents (Eissenberg *et al.* 1990, 1992; Garzino *et al.* 1992; Cléard *et al.* 1997) or as potential heterochromatin constituents (Reuter *et al.* 1990; Tschiersch *et al.* 1994) with dose-dependent opposite effects. One such gene, *Su(var)205*, encodes the heterochromatin-associated protein HP-1 (Eissenberg *et al.* 1990). More recently, *Su(var)3-7* was identified as a nuclear protein that associates with pericentric heterochromatin and coimmunoprecipitates with HP-1, suggesting that the two proteins cooperate in building a silencing complex that is responsible for the genomic silencing associated with heterochromatin (Cléard *et al.* 1997). Moreover, histone modification, such as deacetylation, might affect how tightly the DNA is associated with the histones and/or might be necessary to recruit proteins to establish the silencing (De Rubertis *et al.* 1996).

Direct evidence of altered chromatin structure associated with the PEV phenomenon was first described by Wallrath and Elgin (1995). Using standard chromatin analysis on isolated nuclei, they showed that one particular *hsp26* promoter site inserted into pericentric heterochromatin via *P* elements presents both a reduced accessibility to restriction enzymes and a correlated, strongly variegated phenotype for these lines. Furthermore, micrococcal nuclease digests showed that these inserts were packaged in a more regular nucleosome array than that observed for euchromatic inserts. However, no such difference has been observed for classical PEV, *i.e.*, *white* locus inversions (Hayashi *et al.* 1990; Locke 1993; Schlossherr *et al.* 1994).

Striking structural and functional similarities exist between PEV and the activities of *Polycomb*-group genes (*Pc*-G) in regulating the homeotic complexes. The expression pattern of the homeotic genes of the Antennapedia and Bithorax complexes are initiated early in embryonic development by the gap and pair-rule genes (Bienz and Muller 1995). These genes are expressed transiently, whereas continuous homeotic gene activity is essential for implementing stable determination of cell fates. The *Pc*-G maintains the repressed state in those cells where the homeotic gene was originally inactive (Paro 1993; Pirrotta *et al.* 1995), whereas the *trithorax* group (*trx*-G) sustains the active state in cells where the homeotic gene was originally expressed (Kennison 1995). Genetic and molecular data fit well with

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the fact that *Pc*-G gene products may act in large protein complexes. Combinations of different *Pc*-G mutations have a much more severe phenotype than the additive effect of two single mutations (Jürgens 1985), and haplo-insufficient phenotypes of some mutants can be partially rescued by increasing the copy number of another member (Cheng *et al.* 1994). The Polycomb protein (PC) was found to be associated in a multimeric complex with at least one other member of the *Pc*-G, the PH protein (Franke *et al.* 1992). Furthermore, the roughly 100 polytene-binding sites for PC, PH, and PCL completely coincide and largely overlap with those for PSC, E(Z), and ASX (DeCamillis *et al.* 1992; Franke *et al.* 1992; Rastelli *et al.* 1993; Lonie *et al.* 1994; Carrington and Jones 1996; Sinclair *et al.* 1998a).

Moreover, PC and the Drosophila heterochromatin protein HP-1 share a domain of homology (the chromodomain), and, therefore, it was suggested that *Pc*-G proteins may function by regulating higher-order chromatin structures (Paro and Hogness 1991). This regulation is mediated by *Pc*-G response elements (PREs), *cis*-acting DNA elements that are able to maintain the expression boundaries of homeotic genes in a *Pc*-G-dependent manner (Simon *et al.* 1993). When present in reporter gene constructs in transgenic flies, such elements cause additional PC binding at the insertion site on polytene chromosomes (Zink *et al.* 1991), and they are able to silence neighboring reporter genes in a *Pc*-G-dependent manner (Fauvarque and Dura 1993; Chan *et al.* 1994; Gindhart and Kaufman 1995; Gindhart *et al.* 1995; Zink and Paro 1995). The analogy between the variegated phenotype of such reporter genes and PEV led us to call this phenomenon developmental regulator effect variegation (DREV; Fauvarque and Dura 1993). PREs are thus thought to act as sequences that recruit members of the *Pc*-G and nucleate the formation of *Pc*-G-silencing complexes on target genes (Pirrotta and Rastelli 1994; Orlando and Paro 1995). It was proposed that *Pc*-G complexes function by spreading from PREs over extended chromosomal domains. Therefore, as envisaged for heterochromatic silencing, *Pc*-G complexes may package chromatin into a compact structure that prevents the binding of transcriptional activators to enhancer or promoter sequences (Paro 1990). This model is supported by the findings that PC is associated with the entire inactive region of the BX-C in tissue culture cells (Orlando and Paro 1993) and that GAL4-dependent transcription is inhibited by *Pc* in the *bx* regulatory region of the *Ubx* gene in embryos (McCall and Bender 1996). However, no significant difference in the accessibility of restriction endonucleases has been detected between the active and inactive states of the *Antennapedia* gene in imaginal discs (Schlossherr *et al.* 1994).

The introduction into yeast of the gene encoding the *Escherichia coli* Dam methyltransferase has allowed the study of chromatin structure *in vivo* without the complications of *in vitro* artifacts introduced during extraction or purification procedures. Telomeric sequences, which, in yeast, induce a gene silencing that resembles that of Drosophila PEV, are undermethylated compared to euchromatic sequences (Gottschling 1992). Other more recent studies confirm the ability of exogenous methylases to probe yeast chromatin structure *in vivo* (Singh and Klar 1992; Kladde and Simpson 1994; Fisher-Adams and Grunstein 1995; Kladde *et al.* 1996). As in yeast, the genomic DNA of Drosophila is not methylated to a detectable level (Bird 1992). Wines *et al.* (1996) reported introduction of the *dam* gene into Drosophila. They were unable to detect a significant difference in methylation levels between euchromatic and heterochromatic sites because they compared the accessibility of multiple sites at different loci simultaneously rather than the same site in the two different contexts.

Using a novel strategy, we report here that the *E. coli* Dam methylase is able to detect chromatin variations correlated with gene silencing *in vivo*. Probing the same sequences in different contexts, we show that the accessibility to Dam methyltransferase activity is significantly reduced at the *white* locus in cases of PEV for pericentric *P* elements and for *white* chromosomal rearrangements. Furthermore, using the same experimental design, we report evidence that the DREV phenomenon, *i.e.*, *Pc*-G-mediated repression, is also correlated with a detectable, altered chromatin structure. Regarding the results, these two gene-silencing mechanisms, although based on different protein factors, may involve similar higher-order chromatin structure.

MATERIALS AND METHODS

Drosophila stocks: All Drosophila stocks were raised on standard culture medium. Crosses were performed at 25°. Flies of similar ages were used for all comparisons. All variants used are described in Lindsley and Zimm (1992), Fauvarque and Dura (1993), and Wallrath and Elgin (1995). The *In(1)w*^{m4hrx342} is an X-ray-induced revertant of the *In(1)w*^{m4h} mutant strain (G. Reuter, personal communication). The *ph*$^{w\Delta}$ viable mutant allele was obtained for this study after imprecise excision of the *P* element (Robertson *et al.* 1988) contained in the *ph*$^{lac+3}$ *w*^{1118} stock (Fauvarque *et al.* 1995). White-eyed male derivatives were mated with *ph*$^{lac+3}$ *w*^{1118} females, and the female progeny was checked for the darker eye phenotype. This new *ph* mutant allele was confirmed by demonstrating lethality over a null allele of *ph*. The deficiency *Df(1)w*^{vco}, derived from the *Tp(1;3)w*^{vco} strain, includes the *ph* and *white* loci, and it was chosen as a *ph* mutant background to test the same *Bcl*I sites of the *white* gene as in the PEV studies. As this deficiency is male lethal, females bearing either the wild-type chromosome or one mutant for *ph* (or *Psc*), as well as the *P*[*ph*] insert (either *T30* or *T3*) and the Dam methylase, were analyzed.

The *P*[*y*$^{+}$; *dam*$^{+}$] construct: The *E. coli dam methyltransferase* gene was obtained from pDOX1 (Hülsmann *et al.* 1991); the *dam* gene was subcloned into pBluescript as a *Xho*I-*Sal*I 1027-bp fragment, and then the blunted *Not*I/*Sal*I fragment was subcloned into the transformation vector phy701 (Fauvarque

et al. 1995), cleaved by *Not*I/*Xba*I, and then blunted between the *hsp70* promoter and the polyadenylation site. phy701 is derived from pCaSpeR-hs (Thummel and Pirrotta 1992), whose *white* gene has been replaced by the *yellow* gene from Dint plasmid (Geyer and Corces 1987). Detailed maps of the plasmids are available on request. The use of the *yellow* gene rather than the *miniwhite* gene as the transformation marker avoids interference when probing either sequences of the endogenous *white* for classical PEV studies or sequences of the *miniwhite* transgene contained in *P*-element constructs. This plasmid, named phy705, is designated *P*[y^+; dam^+].

Generation of transgenic flies: phy705 (0.3 mg/ml) was coinjected with helper plasmid pUChsP 2-3 (0.15 mg/ml) into *y* w^{67c23} embryos following standard procedures (Rubin and Spradling 1982). One transformant was recovered with an intact single copy of the *P* element, as checked by genomic Southern blot analysis. Several additional lines were recovered after standard mobilization procedure of the initial *P*[y^+; dam^+] element (Robertson *et al.* 1988).

Chromatin structure analysis: Flies of similar ages grown at 25° without heat shock were used because the basal transcription level of the *hsp70* promoter provides sufficient methylase activity. Before DNA preparation, flies were frozen at −80°. Samples of genomic DNA were extracted from 25 males or from 20 females: typically, flies were homogenized in 0.1 m Tris-HCl, 0.1 m EDTA, and 1% SDS, and incubated at 65° for 30 min. After addition of potassium acetate (1 m final), samples were cooled on ice for 30 min. Supernatants were recovered after two rounds of centrifugation (10 min, 13000*g*, 4°). DNA was precipitated with isopropanol (0.6 volume), washed in 70% ethanol, dried in a Speedvac, and resuspended in 50 μl 10 mm Tris, pH 8, and 1 mm EDTA. The DNA preparation (25 μl) was cleaved to completion with the first enzyme to flank the tested sites. The enzymes used were *Sal*I for the 5′ region and *Csp*6I for the 3′ region of the pericentric *P* inserts, *Bam*HI for the *lacZ* transgene and for the classical PEV studies, *Bgl*II for the PEV-unrelated sequence, and *Hin*dIII for the euchromatic *P*[*ph*] transgenes. The DNA was then cleaved with *Bcl*I at 50° for 5–6 hr, except for the 3′ region of *miniwhite* or *white* studies, in which *Mbo*I was used at 37°. The DNA was size fractionated on a 1% agarose gel and transferred to a reinforced cellulose nitrate membrane. Blots were then hybridized with the appropriate ^{32}P probe, labeled by random priming (Random Prime-IT kit; Stratagene, La Jolla, CA) in 50% deionized formamide, 5× SSPE, 5× Denhardt's, and 0.1% SDS at 42° for 12 hr, then washed three times for 10 min with 3 mm Tris, pH 9, at room temperature. The probes used were the 650-bp *Bcl*I fragment from the 5′ region of the *white* locus (see Figures 1A, 3A, and 5A), a 587-bp PCR-amplified fragment from the 3′ region of the *white* locus (Figure 1B), the 977-bp *Bgl*II-*Bcl*I fragment from the *lio* locus (Figure 3D), and a 650-bp, PCR-amplified fragment from *lacZ* sequence (Figure 2B). Primers used for PCR-amplified probes were, for the 3′ region of *white*, 5′-GCCGCTGGACTACG TGGG-3′ near an *Mbo*I site and 5′-CGAAATGAACCACTCGG AACC-3′ near a *Csp*6I site, and for the *lacZ* study, 5′-CTGGGTCGGTTACGGCCAGG-3′ near a *Sal*I site and 5′-GC ACCATCGTCTGCTCATCC-3′ near an *Eco*RV site. Autoradiograms were visualized on 3M Hyperfilm. Southern blots were scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The relative intensity of each band was calculated by measuring the intensity of the band compared with the total intensity of the bands in a given lane.

Northern analysis: Total RNA was isolated (Sambrook *et al.* 1989) from flies containing the *P*[*UAS-lacZ*] insertion (Brand and Perrimon 1993) with or without the *P*[*daughterless-GAL4*] (Wodarz *et al.* 1995). Total mRNA was purified using the PolyATtract IV System (Promega, Madison, WI) and fractionated on a 2% agarose gel with formaldehyde. After transfer to a nylon membrane and baking for 1 hr at 80° under vacuum, the membrane was probed with a PCR-amplified *lacZ* fragment and with a plasmid containing the *rp49* gene (Wong *et al.* 1981). The intensity of the bands was quantified using a PhosphorImager (Molecular Dynamics). Differences in the amount of mRNA loaded in each lane were corrected using the signal intensity of *rp49*.

RESULTS

Expressing Dam methyltransferase in flies: Expression of the *E. coli* Dam methylase in Drosophila did not cause detectable defects, neither developmental nor fertility related, in accordance with previous work (Wines *et al.* 1996). Eight *P*[y^+; dam^+] lines were tested for their ability to methylate DNA (Table 1). We had previously checked that DNA extracted from wild-type flies lacking methylase activity was completely cleaved by *Bcl*I (data not shown). All experiments were carried out at 25° without heat shock; the basal transcription level of the *hsp70* promoter provided sufficient methylase activity. Wild-type Canton-S females were mated to males carrying different *P*[y^+; dam^+] insertions. In the case of autosomal *P*[y^+; dam^+] insertions, the male progeny were analyzed. For *X* insertions, the heterozygous females were tested. To assess the activity of the methylase, we have used the *Bcl*I restriction enzyme, which is sensitive to the state of methylation of its TGATCA target site: if this sequence is methylated on the first adenine residue by the Dam activity, *Bcl*I does not cut it. Genomic DNA was first cleaved with *Bam*HI, which is insensitive to methylase, to flank the two *Bcl*I sites of the 5′ region of the *white* sequence (see Figure 3A). DNA was then treated with *Bcl*I. The fraction of DNA uncut by *Bcl*I, probed by the 650-bp *Bcl*I fragment, and measured by a PhosphorImager directly reflects the level of the *in vivo*-methylated DNA. In this case, a combination of four bands was obtained, and the relative intensity of each band was calculated by measuring the intensity

TABLE 1

Methylation level of eight lines bearing a *P*[y^+; dam^+]

Lines[a]	Chromosomal localization	Level of methylation (% ± SE)
dam3	*X*	21.8 ± 0.6
dam10	*X*	41.9 ± 3.6
dam11	*II* (CyO)	23.8 ± 0.2
dam12	*II* (CyO)	31.4 ± 0.3
dam34	*II* (CyO)	35.0 ± 3.1
dam19	*III*	34.7 ± 3.1
dam21	*III*	27.9 ± 0.6
dam27	*III*	51.2 ± 1.0

[a] Genotypes of tested flies were w^{m4}/y w^{67c23} *P*[y^+; dam^+] females for insertions on the *X* and w^{m4}/Y; *P*[y^+; *dam*]/+ males for autosomal insertions.

of one band compared with the total intensity of the bands in a given lane. Accessibility of the more proximal *Bcl*I site was given by the sum of the relative intensity of the 3-kb band, which results from methylation of both sites on the same DNA fragment, and the 1.2-kb band, where only the site proximal to the probe was methylated (see Figure 3A). Results are shown in Table 1. Dam activity ranged from 21.8 to 51.2% on this site. This seems to reflect a position-dependent activity regarding the insertion site of the $P[y^+; dam^+]$. Without heat shock, transcription of the *dam* gene could reflect neighboring enhancer activity, with the level of activity depending on the strength of the enhancer. However, it seems that 50% of methylated DNA is the upper limit that can be obtained in our experiments, as it was for Wines *et al.* (1996). Nucleosomal conformation of DNA, nucleosome phasing, repair system modification of "damaged" methylated DNA, or some demethylase system may explain this upper limit.

The $y\ w^{67c23}$;$P[y^+; dam^+]^{34}$,*CyO*/+ strain (named *dam 34*) was chosen for further tests as the $P[y^+; dam^+]$ was easily monitored by the *CyO* marker.

Measure of accessibility of *miniwhite* sites in heterochromatic *P* elements: Direct evidence of altered chromatin structure in the PEV phenomenon was first described by Wallrath and Elgin (1995). Using standard chromatin analysis on isolated nuclei, they showed that one particular *hsp26* restriction site presents a reduced accessibility to restriction enzyme in *P* elements inserted into pericentric heterochromatin (lines 39C-4 and 118E-12 have an insert at the base of chromosome arms *2*L and *3*R, respectively) compared to the accessibility of the same site localized in a euchromatic position (lines 39C-X and 118E-X have an insert at cytological regions 2D and 19A-B, respectively). The eye phenotypes of flies containing the euchromatic *P* element are wild type, while the eye phenotypes of flies containing heterochromatic *P* element are strongly variegated. An example is shown in Figure 4B (39C-4). Using Dam activity *in vivo*, two new sites of these transposons, one in the 5′ region and the other in the 3′ region of the *miniwhite* sequence, were tested (Figure 1A). To ensure that endogenous *white* sequences could not interfere with the studies, experiments for the 5′ site were performed in a w^{67c23} background, which is deficient for the 5′ region of the *white* locus, including the sequence

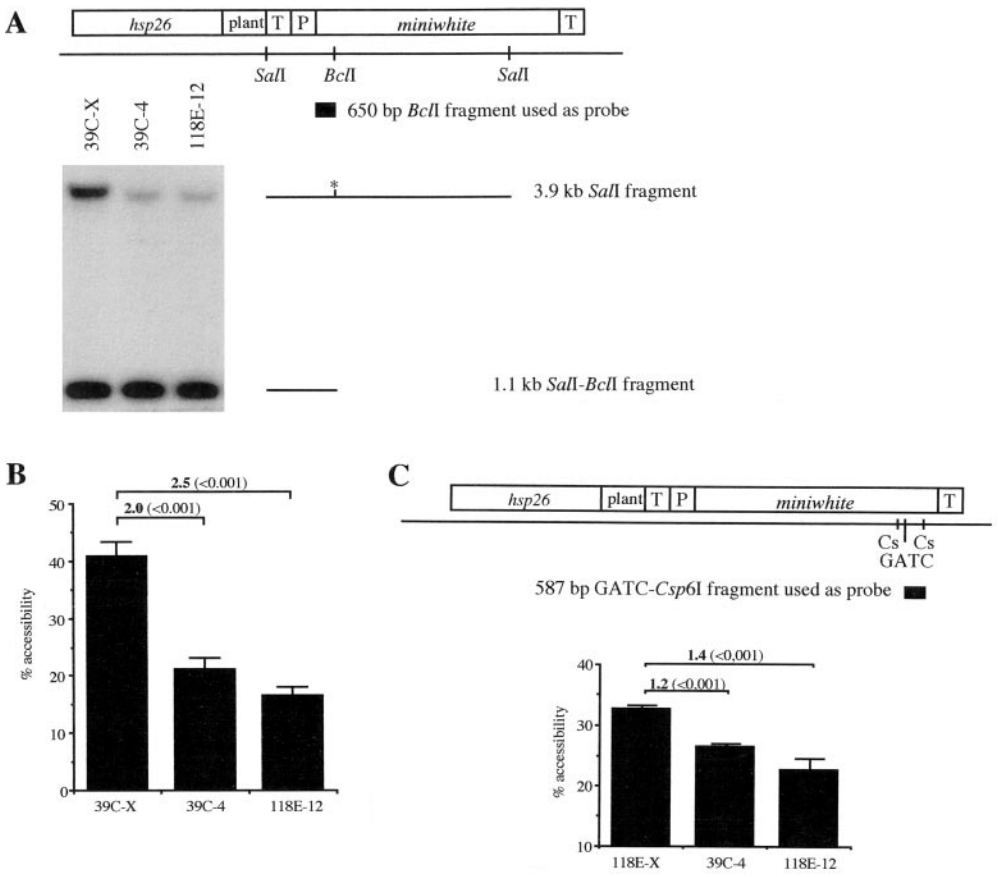

Figure 1.—Methylation levels at the *miniwhite* locus relative to the PEV phenomenon. Heterochromatic *P* transgenes were compared to euchromatic ones. (A) A schematic map of the *P* element tested (Wallrath and Elgin 1995) is given as the corresponding DNA fragments obtained after a *Sal*I-*Bcl*I double digest. A typical Southern blot analysis shows the variation of the proportion of the methylated fragment between a uniformly expressed euchromatic line (39C-X) and variegated ones (39C-4 and 118E-12). (B) Relative intensity of a methylated fragment quantified with a PhosphorImager. Numbers over square brackets indicate the fold-factor difference between two lines (in bold type) and the probability that the difference observed, as determined by the Student's statistical test, results by chance (in parentheses). Values are 40.7% ± 2.7 ($n = 6$) for 39C-X hemizygous males, 21.1% ± 2.0 ($n = 6$) for 39C-4 heterozygous males, and 16.5% ± 1.5 ($n = 6$) for 118E-12 heterozygous males. (C) The sites in the 3′ region assayed for accessibility are given on the schematic map of the *P* transgene. Cs is an abbreviation for *Csp*6I, and GATC is the tested site, which is cleaved by *Mbo*I. Values are 32.6% ± 0.5 ($n = 6$) for 118E-X hemizygous males, 26.5% ± 0.4 ($n = 6$) for 39C-4 heterozygous males, and 22.7% ± 1.7 ($n = 7$) for 118E-12 heterozygous males.

corresponding to the 650-bp *Bcl*I fragment used as probe. Similarly, experiments for the 3′ site were performed in a w^{IR8} background, which is deficient for the 3′ region of *white*, including the 587-bp *Mbo*I-*Csp*6I fragment used as probe. In these cases, only one *Bcl*I site or *Mbo*I site was tested between flanking sites—*Sal*I and *Csp*6I, respectively—and a simple combination of two bands was observed. Accessibility was measured as the intensity of the upper band compared to the total intensity of the two bands in a given lane. This relative quantification is independent of the quantity of DNA loaded in each lane. The results for the 5′ and 3′ regions (Figure 1, B and C), show a significant decrease in the level of methylation for the heterochromatic inserts compared to the euchromatic ones. For the 5′ site, 2.5- and 2.0-fold factors were observed between the accessibility of the euchromatic line 39C-X and the variegating lines 118E-12 and 39C-4, respectively. A lower amount of material in lanes 39C-4 and 118E-12 may explain why the 1.1-kb product does not increase while a reduction of the corresponding 3.9-kb fragment is observed. In the 3′ region, the differences were less pronounced, as 1.4- and 1.2-fold factors were observed between the euchromatic line 118E-X and the variegating lines 118E-12 and 39C-4, respectively. This is consistent with the lesser accessibility expected for those heterochromatic inserts. Dam activity *in vivo* correlates with previous results based on standard chromatin analysis using isolated nuclei. The fact that both sites in the 5′ and 3′ regions of *miniwhite* exhibit a reduction of the level of methylation when localized into heterochromatin, as with the previous study by Wallrath and Elgin (1995) on the *Xba*I site in the *hsp26* promoter region, suggests strongly that the general accessibility of the pericentric *P* elements is reduced.

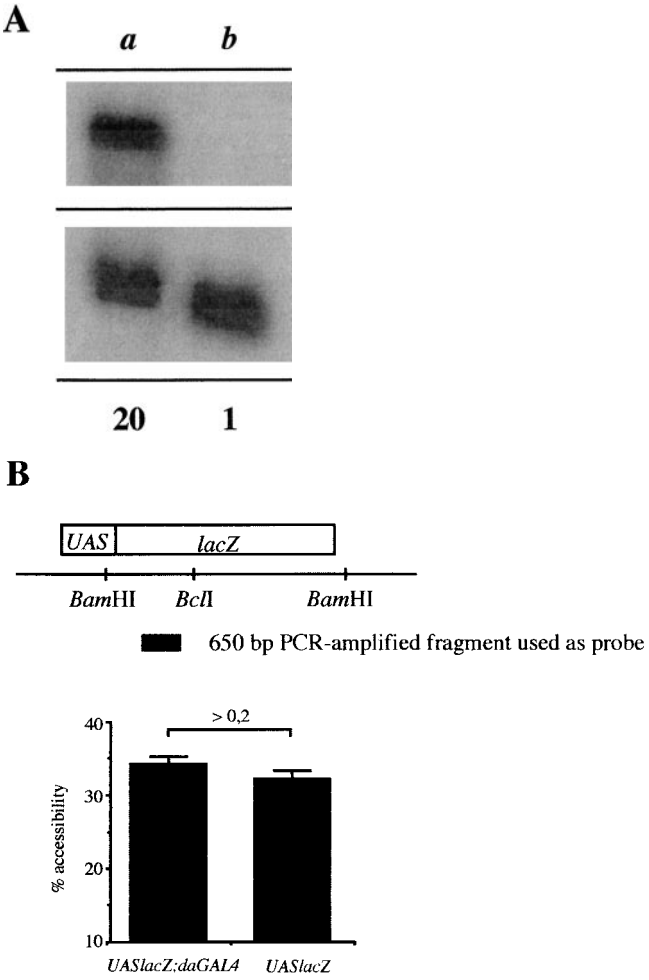

Figure 2.—Relation between Dam activity and transcriptional activity. (A) Northern blot analysis of *lacZ* transcription. (Lane a) mRNA extracted from heterozygous flies containing both *P*[*UASlacZ*] (Brand and Perrimon 1993) and *P*[*daGAL4*] (Wodarz *et al.* 1995). (Lane b) mRNA extracted from heterozygous flies containing only the *P*[*UASlacZ*]. (Upper panel) *lacZ* probe. (Lower panel) *rp49* probe. Numbers indicate ratios of *lacZ* transcripts after normalization with the *rp49* signal. (B) A schematic map of the *P*[*UASlacZ*] element and the probe used for this experiment. Diagram shows the relative intensity of the methylated fragment. Values are 32.9% ± 1.1 ($n = 9$) for *P*[*UASlacZ*];*P*[*daGAL4*] and 30.1% ± 1.2 ($n = 9$) for *P*[*UASlacZ*] heterozygous flies, and they are not significantly different ($P > 0.2$).

Effect of transcriptional activity on methylation: To examine whether the level of methylation *in vivo* might reflect only transcriptional activity rather than a higher-order chromatin structure that is independent of the transcriptional apparatus, we have checked the accessibility of one site in a *lacZ* sequence driven by the UAS activation sequence. Without the *GAL4* activator, no *lacZ* transcript was detectable on a Northern blot (Figure 2A). However, with the *GAL4* activator under the control of the ubiquitous *daughterless* promoter (Wodarz *et al.* 1995), transcription of *lacZ* was enhanced by at least 20-fold (see materials and methods). A 650-bp *lacZ* fragment was used to probe the accessibility to methylase activity of genomic DNA of flies containing *P*[*UAS lacZ*] (Brand and Perrimon 1993) and *P*[y^+; dam^+] in the presence or absence of the *GAL4* activator. No significant difference in the level of methylation on the *lacZ* sequence was detected whether or not transcription of *lacZ* was activated (Figure 2B). This result suggests that the transcriptional activity does not modify accessibility measured by Dam activity *in vivo*. We, therefore, conclude that differences of accessibility observed for transposons in heterochromatin *vs.* euchromatic environments reflect some higher-order modification of the chromatin structure.

Rearrangement of the *white* locus near heterochromatin is associated with a reduced DNA accessibility: The wild-type strain Canton-S, variegating lines $In(1)w^{m4}$ and $In(1)w^{m4h}$, and the revertant line $In(1)w^{m4hrx342}$ derived from $In(1)w^{m4h}$ were assayed on the two *Bcl*I sites of the 5′ region of *white* (Figures 3A and 4, A and D, for eye phenotype). As two *Bcl*I sites were simultaneously tested, a combination of four fragments was observed. Similarly to the proximal site described previously, accessibility

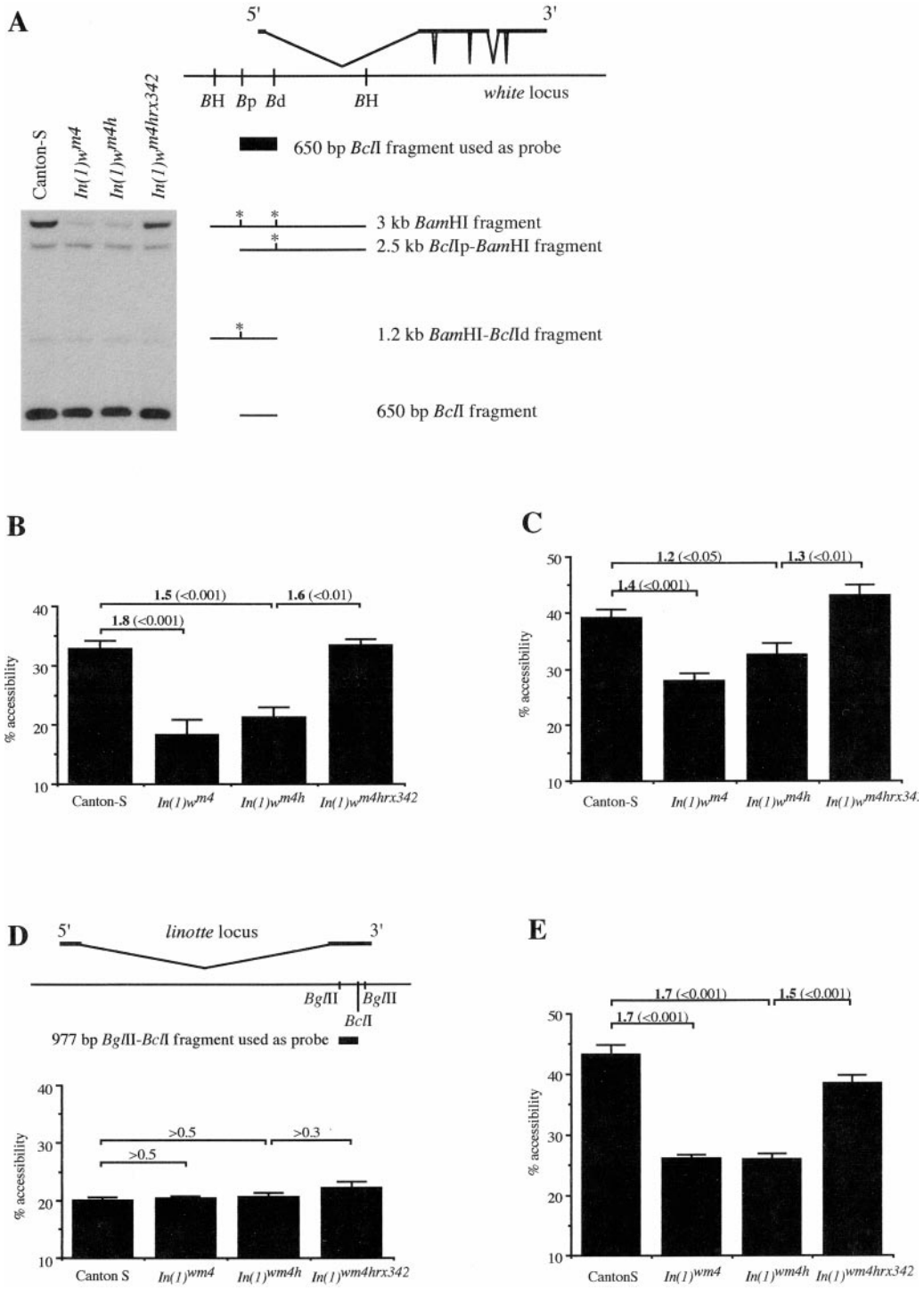

Figure 3.—Methylation levels at the *white* locus relative to the PEV phenomenon. (A) A schematic map of the *white* locus is given as the corresponding DNA fragments obtained after double digest *Bam*HI-*Bcl*I. *B*H is for *Bam*HI, *B*p is for the proximal *Bcl*I site, and *B*d is for the distal *Bcl*I site. A typical Southern blot analysis shows the variation of the proportion of methylated fragments between wild-type (Canton-S/*Y*), variegated [*In(1)w*^{m4}/*Y* and *In(1)w*^{m4h}/*Y*], and revertant males [*In(1)w*^{m4hrx342}/*Y*]. (B) Relative intensity of methylated fragment for the proximal *Bcl*I site. Values are 32.8% ± 1.4 (n = 7) for Canton-S/*Y* males, 18.3% ± 2.5 (n = 5) for *In(1)w*^{m4}/*Y* males, 21.2% ± 1.8 (n = 5) for *In(1)w*^{m4h}/*Y* males, and 33.4% ± 1.1 (n = 4) for *In(1)w*^{m4hrx342}/*Y* males. (C) Relative intensity of methylated fragment for the distal *Bcl*I site. Values are 39.0 ± 1.7 (n = 7) for Canton-S/*Y* males, 27.8% ± 1.4 (n = 5) for *In(1)w*^{m4}/*Y* males, 32.5% ± 2.0 (n = 5) for *In(1)w*^{m4h}/*Y* males, and 43.1 ± 2.0 (n = 4) for *In(1)w*^{m4hrx342}/*Y* males. (D) A schematic map of the PEV-unrelated *linotte* locus and the probe used. Relative intensity of methylated fragment for the *Bcl*I site at this locus. Values are 20.0% ± 0.6 (n = 4) for Canton-S/*Y* males, 20.4% ± 0.3 (n = 4) for *In(1)w*^{m4}/*Y* males, 20.6 ± 0.7 (n = 4) for *In(1)w*^{m4h}/*Y* males, and 22.2% ± 1.0 (n = 4) for *In(1)w*^{m4hrx342}/*Y* males, and they are not significantly different, as P is never <0.3. (E) Relative intensity of the methylated fragment for the distal *Bcl*I site of the *white* locus in *X*O males. Values are 43.3% ± 1.6 (n = 5) for Canton-S/*Y* males, 26.1% ± 0.6 (n = 5) for *In(1)w*^{m4}/*Y* males, 25.9% ± 1.0 (n = 7) for *In(1)w*^{m4h}/*Y* males, and 38.5% ± 1.4 (n = 11) for *In(1)w*^{m4hrx342}/*Y* males.

of the *Bcl*I-distal site was given by the sum of the relative intensities of the 3- and 2.5-kb bands where only the distal site was methylated. Variegating lines showed a lower methylation level for both sites than did the wild-type and revertant strains (Figure 3, B and C). For the more proximal site, Canton-S differs from *In(1)w*^{m4} and *In(1)w*^{m4h} by 1.8- and 1.5-fold, respectively. For the distal site, subtle but significant differences remain: 1.4- and 1.2-fold factors were observed between the same strains. The *In(1)w*^{m4hrx342} strain presents essentially the same accessibility level as the wild type, showing that the relocalization of the *white* locus outside the heterochromatin region is correlated with the return of a "wild-type" chromatin conformation.

Similar experiments were also carried out with the *dam27* line (see Table 1). Although the activity of the

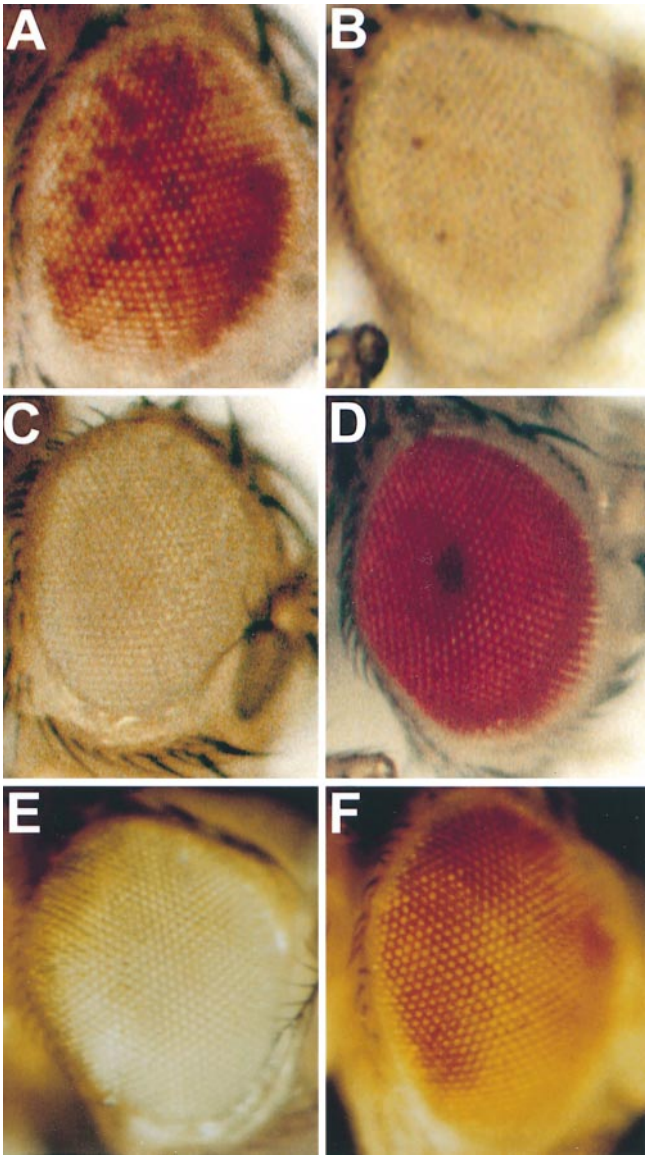

Figure 4.—Eye phenotype of some of the strains used in this study. (A) *In(1)w*^{m4h}/*Y* male. (B) 39C-4/+ male. (C) *In(1)w*^{m4h}/0 male. (D) *In(1)w*^{m4hrx342}/*Y* revertant male. (E) +/+;T30/T30 female. (F) *Psc*^{1}/+;T30/T30 female.

Dam enzyme in this line was higher, the difference remains similar: for the more proximal site, Canton-S differs from *In(1)w*^{m4} and *In(1)w*^{m4h} by 1.8- and 1.6-fold, respectively. For the distal site, 1.4- and 1.2-fold factors were observed between the same strains (data not shown). This result shows that the whole DNA of the fly is not saturated by methylation. Nonmethylated DNA resulting from a lack of Dam activity is considered an inaccessible conformation by our test. Therefore, higher Dam activity enhances both wild-type and mutant accessibility. As a consequence, the same ratio of methylated DNA in experimental *vs.* control samples was observed regardless of the activity of the expressing Dam strain used.

As for transposon studies, localization near or in heterochromatic regions seems to be correlated with a higher-order chromatin compaction. One can note that in the case of inversion near heterochromatin of the entire *white* locus and its surrounding euchromatin, the differences observed are less pronounced than in the case of smaller transposons, *i.e.*, 10 kb of euchromatic DNA inside heterochromatin.

Figure 3E shows the effect of the lack of the *Y* chromosome, a strong enhancer of PEV (compare Figure 4, A and C). In *XO* Canton-S males, the proximal site exhibits 1.9- and 2.0-fold differences with *In(1)w*^{m4}/0 and *In(1)w*^{m4h}/0, respectively (data not shown). For the distal site, differences were slightly enhanced as a 1.7-fold factor was observed between Canton-S and each variegating line.

Line specificity cannot account for the accessibility difference: Contrary to transposon line studies where all inserts are in the same genetic background, PEV lines could potentially have particularities because of their own specific genetic background. To test this possibility, a PEV-unrelated euchromatic site in 37D at the *linotte* locus (Dura *et al.* 1995) was assayed for methylation level for each PEV line (Figure 3D). If modifiers of Dam activity are present in the genetic background of tested lines, corresponding variations would be expected at this locus. In fact, no significant difference was detected, indicating that strain specificity cannot account for the accessibility differences observed above.

Dam activity reveals chromatin alteration in *Pc*-G-mediated silencing: Using the same experimental design, namely assaying the accessibility of the *Bcl*I sites contained in the *white* sequence, we tested the role of the *Polycomb* group gene products on the euchromatic PRE-induced variegation (DREV). Chromatin accessibility assays were performed on the *miniwhite* gene of two euchromatic *P*[*ph*] inserts, T30 and T3 (Fauvarque and Dura 1993), respectively located in 86CD, which is a binding site of the PC/PH complex on the polytene chromosome, and 65F, which is not a site of binding of the complex (Zink and Paro 1989). In these constructs, a *ph* regulatory region that induces variegation lies upstream of *miniwhite* driven by its own promoter. This variegation is not sensitive to classical PEV modifiers, but it is strongly suppressed in *ph* (see Figure 5 in Fauvarque and Dura 1993) and *Psc*^{1} (Figure 4, E and F) mutant backgrounds. The results (Figure 5A and 5B) show a significant rise in accessibility in derepressed mutants (*ph* and *Psc*) compared to the wild-type control for both tested inserts. For the more proximal 5′ *miniwhite Bcl*I site of the T30 insert, a 2.1-fold difference was obtained between a wild-type background and the homozygous *ph*$^{w\Delta}$ context. In a *Psc*^{1} or heterozygous *ph* deficiency background, a 1.6-fold factor was observed. A similar derepression is observed for the distal *Bcl*I site, as a 1.9-fold factor was obtained between +/+ and *ph*$^{w\Delta}$/*ph*$^{w\Delta}$ flies, and a 1.5-fold factor was obtained between wild type and *Psc*^{1} or *ph* deficiency. One can note that variations are stronger in the case of the T30 insertion than for the T3 *P* element, where the difference never exceeded 1.5-fold. The PEV-unrelated *lio*

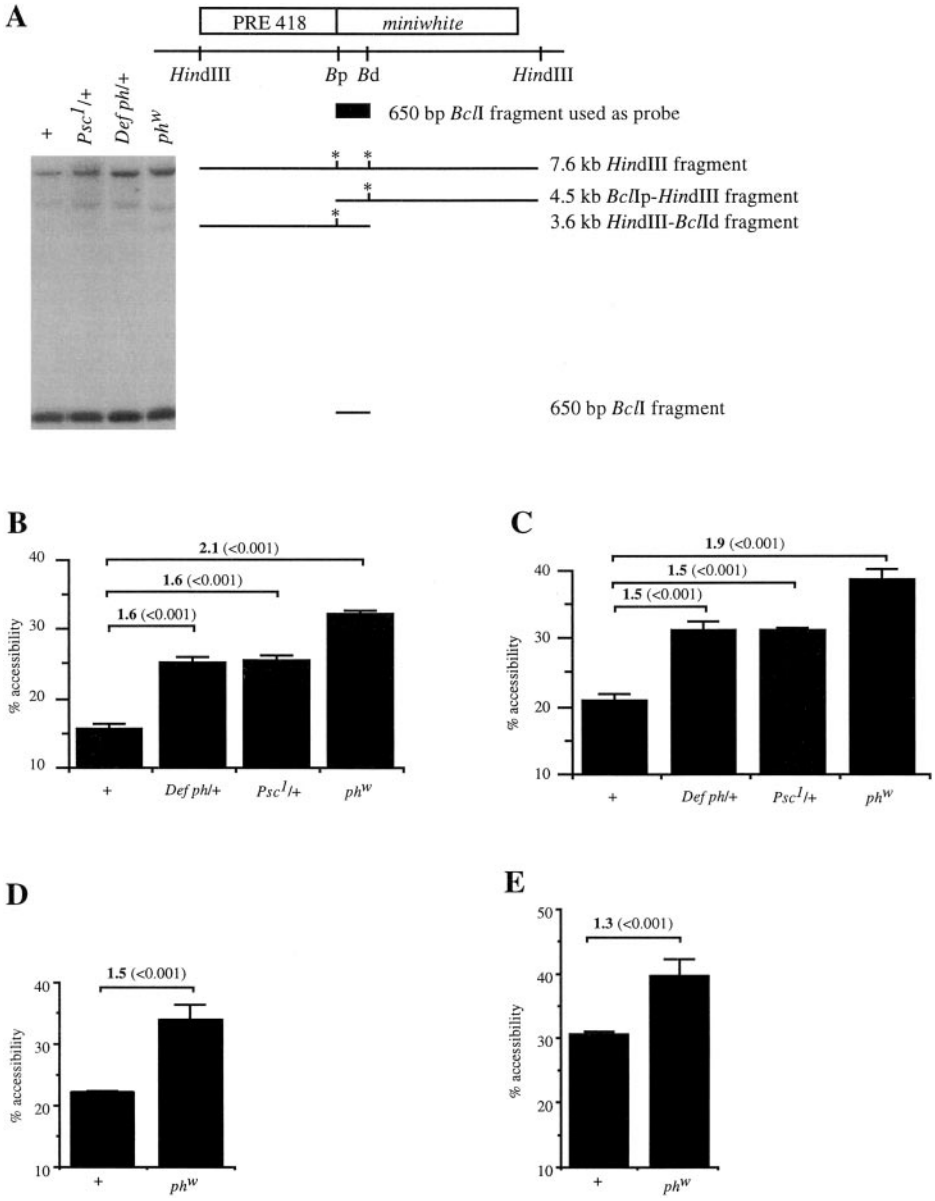

Figure 5.—Methylation levels at the *miniwhite* locus relative to the DREV phenomenon. (A) A schematic map of the *P*[*ph*] (Fauvarque and Dura 1993) and the DNA fragments obtained after double-digest *Hin*dIII-*Bcl*I is given. A typical Southern blot analysis shows the variation of the proportion of methylated fragment in different genetic backgrounds. + corresponds to $y\ w^{67c23}/y\ w^{67c23}$, $Psc^1/+$ corresponds to $y\ w^{67c23}/y\ w^{67c23};Psc^1/+$, *Def ph*/+ to $Df(1)w^{vco}/y\ w^{67c23}$, and $ph^{w\Delta}$ corresponds to $y\ ph^{w\Delta}/y\ ph^{w\Delta}$. (B) Relative intensity of the methylated fragment for the proximal *Bcl*I site of the T30 insert. Values are 15.5% ± 0.9 ($n = 5$) for +, 25.1% ± 0.9 ($n = 6$) for *Def ph*/+, 25.5% ± 0.7 ($n = 6$) for $Psc^1/+$, and 32.0% ± 0.7 ($n = 4$) in case of $ph^{w\Delta}$. (C) Relative intensity of methylated fragment for the distal *Bcl*I site of the T30 insert. Values are 20.6% ± 1.0 ($n = 5$) for +, 30.9% ± 1.3 ($n = 6$) for *Def ph*/+, 30.8% ± 0.4 ($n = 6$) for $Psc^1/+$, and 38.2% ± 1.5 ($n = 4$) for $ph^{w\Delta}$. (D) Relative intensity of methylated fragment for the proximal *Bcl*I site of the T3 insert. Values are 22.1% ± 0.2 ($n = 8$) for the + and 33.9% ± 2.5 ($n = 8$) for $ph^{w\Delta}$. (E) Relative intensity of methylated fragment for the distal *Bcl*I site of the T3 insert. Values are 30.6% ± 0.4 ($n = 8$) for + and 39.6% ± 2.7 ($n = 8$) for $ph^{w\Delta}$.

sequence was also assayed for accessibility to know if a *ph* mutant background can influence Dam activity. No differences were observed: compare 15.4% ± 0.5 ($n = 4$) in a wild-type background to 15.1% ± 0.5 ($n = 4$) in the *ph* deficiency context. This indicates that methylase activity does not change in a *ph* mutant background.

DISCUSSION

Probing Drosophila chromatin structure *in vivo* with the *E. coli* Dam DNA methylase activity: Using the *E. coli* Dam DNA methylase activity, we were able to show that a significantly reduced accessibility is detected at one specific *Bcl*I site at the *miniwhite* sequence of the pericentric *hsp26* transgenes compared to euchromatic control transgenes. This result shows conclusively that the adult Drosophila chromatin structure can be assayed directly *in vivo*. Furthermore, Dam activity revealed that both the 5′ and 3′ regions of the gene were less accessible in the case of pericentric location, compared to euchromatic controls. This result indicates that the use of Dam is able to corroborate *in vivo* the previous results obtained by standard chromatin analysis (Wallrath and Elgin 1995), and that the whole *P*-element DNA seems to be in a "closed" chromatin conformation, entirely wrapped with heterochromatin proteins and consistent with the model of chromatin compaction (Zuckerkandl 1974; Locke *et al.* 1988).

Heterochromatin has been defined as those regions of the chromosomes that remain condensed throughout the cell cycle and that are associated with the absence of gene expression. The long-held hypothesis that this gene silencing is associated with an altered chromatin structure received direct evidence only recently

(Wallrath and Elgin 1995). These authors reported a standard chromatin analysis using isolated nuclei in which heterochromatic insertions of *P* elements (*hsp26* transgenes) were correlated with a lower accessibility to a specific restriction site than the control euchromatic *P* element. The overall nucleosomal organization showed a more packaged array than the controls did. There have been a few earlier studies of the chromatin structure in classical rearrangements giving rise to PEV (w^{m4} inversion). A DNAse I digestion study using nuclei isolated from third instar larvae was unable to detect any difference in the packaging of DNA at the *white* locus, even though the relocation of the gene near the heterochromatin was correlated with dramatic differences in chromosomal morphology (Hayashi *et al.* 1990). Monitoring the resistance of the heterochromatin-euchromatin junction fragment to degradation by endogenous nucleases showed that the w^{m4} inversion did not induce significant effects on chromatin structure (Locke 1993). Using the ligation-mediated PCR procedure on nuclear extracts, no significant differences on chromatin accessibility were detected on the *white* locus, in a w^{m4} chromosome, in the presence of either a strong suppressor or a strong enhancer of PEV (Schlossherr *et al.* 1994).

Following the same experimental scheme as for the transgenes study, we used the *E. coli* Dam DNA methylase for studying classical PEV. Significant variations can be detected on the two tested sites of the 5′ region of the *white* locus. Moreover, the difference between control and variegated lines is greater for the proximal *Bcl*I site than for the distal one. This may reflect each site having its own accessibility level to the Dam methylase, perhaps because of a particular nucleosomal conformation. Those differences of accessibility were the first described at the *white* locus itself, as previous reports related no detectable difference between heterochromatic sites *vs.* euchromatic ones. The variations we detected at the *white* locus were subtle, and it is possible that the classical techniques requiring chromatin purification before an accessibility test sustain damage to an unstable chromatin architecture. Our method allows the reliable detection of differences of the magnitude twofold or less, simply and reproducibly.

On the basis of our own data, we believe that the contrasting published results of the *hsp26* and the w^{m4} studies reflect differences in the sensitivity of the techniques rather than in the mechanisms inducing PEV. The effects seen in our experiments are stronger with the *hsp26* transgenes than with w^{m4}. This difference might result from the fact that in the variegated rearrangement, a large block of euchromatin is brought into juxtaposition with a block of heterochromatin, whereas in the case of the *hsp26* transgene, a ±10-kb fragment of euchromatic DNA has been inserted within heterochromatin. One can note that in these two cases of PEV, the chromatin compaction, revealed by the *in vivo* Dam methylation, seems correlated with the strength of the variegated phenotype (compare Figure 4, A and B). However, only a slight increase of compaction was observed when w^{m4h} males lacking the *Y* chromosome were tested, although a clear increase of *white* repression was phenotypically observed (compare Figure 4, A and C).

Like PEV, the DREV phenomenon is correlated with a lesser DNA accessibility *in vivo*: The repression of gene expression by the *Polycomb*-group gene products seems, in many aspects, similar to PEV. Again, using the same experimental scheme, we tested the role of the *Polycomb* group gene products on the euchromatic PRE-induced variegation. Fauvarque and Dura (1993) reported that euchromatic transgenes containing the *miniwhite* sequences and a *ph* PRE (*P*[*ph*]) display variegation that is sensitive to some *Pc-G* mutations. Testing the accessibility of the Dam activity to the same sites of *miniwhite* as those tested in the PEV studies of two of these transgenes, we have shown that in a *ph* or in a *Psc* mutant background, an enhancement of accessibility of these sites is correlated with the derepression of *miniwhite* expression observed in the eye. The strongest differences observed in the case of DREV, in a homozygous *ph* mutant background, are similar to those obtained in *hsp26* transposons studies. This led us to think that the chromatin fiber that occurred in DREV might be as "closed" by compaction as for strong PEV. Although there may exist some proteins playing a role in both of these gene silencing mechanisms, such as E(z) (Laible *et al.* 1997), E(Pc) (Sinclair *et al.* 1998b), and ASX (Sinclair *et al.* 1998b), little overlap has been noted between the proteins involved (Fauvarque and Dura 1993; Fauvarque *et al.* 1995; Gindhart and Kaufman 1995; Kennison 1995).

One model has been proposed to explain gene silencing where the chromatin fiber is left unaltered, but the region to be silenced is assigned to a compartment within the nucleus to which not all transcription factors have access (Paro 1993; Schlossherr *et al.* 1994). If the Dam methylase is also excluded from this compartment, then it is clear that the use of this tool cannot discriminate between the "compartmentalization" model and the packaging model.

Moreover, in a review, Simon (1995) schematizes the two extreme packaging models that can be elaborated, based on the available data, for stable repression by *Pc*-G proteins. In the first, the enhancer interference model, the *Pc*-G proteins coat only the PREs preventing, in one way or another, transcription [see also Pirrotta and Rastelli (1994) for another form of a similar model]. In the second, also called the chromatin accessibility model (adapted from Paro 1990), the *Pc*-G proteins coat the entire region of DNA to be inactivated. Our results can exclude the first model because differences of chromatin accessibility are detected outside the PREs. On the other hand, they fit well with the

finding that, after formaldehyde cross-linking in fly tissue culture cells, the PC protein is associated continuously across tens of kilobases in regions that are transcriptionally silenced in the *bithorax* complex (Orlando and Paro 1993). Moreover, a similar, improved method has shown that the PC protein is not distributed homogeneously along the bithorax complex, but that it spreads locally over a few kilobases of DNA surrounding PREs (Strutt *et al.* 1997). This is in good correlation with our results because the two tested sites in the *mini-white* gene are <1 kb away from the PRE.

It would be interesting to assay the question of compaction during development. Preliminary tests on embryos and third instar larvae were negative, as the Dam was expressed at too low a rate to detect variation in methylation ratio.

McCall and Bender (1996) examined the accessibility of DNA-binding proteins for DNA of the BX-C that was under *Pc*-G repression. They found that GAL4-dependent transcription is inhibited by *Pc*, whereas T7 polymerase-dependent transcription is not. This apparent contradiction is likely a result of the small size of the T7 polymerase because an enlarged T7 polymerase (T7-β-galactosidase fusion) is blocked by *Pc* in early embryos (D. Fitzgerald and W. Bender, personal communication). Thus, one means for reducing the background level of DNA methylation may be to engineer a bigger probe, *i.e.*, a fusion of β-galactosidase protein with the methylase. This might be a way to limit the access of the Dam to the chromatin and, therefore, enhance the site difference accessibility between an active and inactive gene in PEV and DREV phenomena.

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