Chromosoma (1996) 104:332–340

CHROMOSOMA

Introduction of a DNA methyltransferase into *Drosophila* to probe chromatin structure in vivo

Debora R. Wines*, Paul B. Talbert, Denise V. Clark, Steven Henikoff**

Howard Hughes Medical Institute, Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98104, USA

Received: 6 June 1995; in revised form: 10 August 1995/Accepted: 25 August 1995

Abstract. The *dam* DNA methyltransferase gene from *Escherichia coli* was introduced into *Drosophila* in order to probe chromatin structure in vivo. Expression of the gene caused no visible defects or developmental delay even at high levels of active methylase. About half of each target site was found to be methylated in vivo, apparently reflecting a general property of chromatin packaged in nucleosomes. Although site-specific differences were detected, most euchromatic and heterochromatic sites showed comparable degrees of methylation, at least at high methylase levels. Methylase accessibility of a *lacZ* reporter gene subject to position-effect variegation throughout development was only slightly reduced, consistent with studies of chromatin accessibility in vitro. Silencing of *lacZ* during development differed from silencing of an adjacent *white* eye pigment reporter gene in the adult, as though chromatin structure can undergo dynamic alterations during development.

Introduction

Flies and yeasts have no known covalent DNA modifications (Bird 1992). Nevertheless, methylases that modify DNA have been introduced into the yeast *Saccharomyces cerevisiae* (Hoekstra and Malone 1985). As a result, it has been possible to study chromatin structure in vivo (Singh and Klar 1992; Gottschling 1992; Kladde and Simpson 1994) without the complication of in vitro artifacts introduced during lysis, extraction or purification procedures. To test whether a similar approach could be carried out in *Drosophila*, we chose *Escherichia coli* Dam methyltransferase, which modifies the N^6 position of adenine in the sequence GATC (Brooks et al. 1983). Here we report the first example of a DNA modification in *Drosophila* and the first artificial DNA modification in a multicellular eukaryote. Introduction of the *dam* gene into *Drosophila* and induction of its expression to high levels has no detectable effect on development. We use this in vivo probe to investigate chromatin accessibility, and to examine possible differences between euchromatin and heterochromatin.

Materials and methods

Plasmid construction. The *dam* methyltransferase gene of *E. coli* was obtained from YCpGAL-EDAM (M.K. Raghuraman, personal communication); this plasmid has the *Hind*III-*Pvu*II *dam* fragment from pMFH1 (Hoekstra and Malone 1985) replacing the *HO* gene in pGAL-HO (Jensen and Herskowitz 1984). The *dam* gene was subcloned using the polymerase chain reaction. One primer (5'-CGGAATTCAACATGAAGAAAAATCGCGC-3') was chosen to produce an *Eco*RI site and a consensus ribosome binding site immediately before the translation start site of *dam* at nucleotide 195 (Brooks et al. 1983). The second primer (5'-GCTCTAGATTGATCCGCTTCTCCTTG-3') was chosen to produce an *Xba*I site 23 bp downstream from the *dam* stop codon at nucleotide 1029. The fragment was amplified using *Pfu* polymerase (Stratagene), cleaved with *Eco*RI and *Xba*I, and gel purified. It was then ligated into pUAST (Brand and Perrimon 1993), downstream of five tandemly arrayed GAL4 binding sites linked to the *hsp70* TATA box, yielding a pDAM plasmid.

Germ-line transformation. Except as noted, the *Drosophila* mutations have been described (Lindsley and Zimm 1992). Co-injection of pDAM and helper plasmids into w^{1118} embryos and selection for transformants with eye pigmentation followed standard procedures (Brand and Perrimon 1993; Spradling 1986). For Me1 and Me2, transposons are present on chromosome 2 and are associated with recessive lethality, whereas Me3 on the *X* chromosome and Me4 on chromosome *3* are homozygous viable insertions. *CyO*/HS-*GAL4* and Me/HS-*GAL4* siblings were generated as progeny of Me/*CyO* × HS-*GAL4*/HS-*GAL4*. Heat shock treatments were for 30 min in a 37°C incubator daily beginning 3 days after the 2 day egg-laying period and continuing until eclosion. The HS-*GAL4* line 2–1 used in these studies was kindly provided by Karen Blochlinger.

* *Present address:* Biological and Physical Sciences Department, Montana State University, 1500 N. 30th St., Billings, MT 59101–0298, USA

** *Present address:* Department of Biology, University of New Brunswick, Fredericton, NB, Canada E3B 6E1

Edited by: A. Spradling

Correspondence to: S. Henikoff

Methylase assays. Three days after eclosion, flies were rapidly frozen in liquid nitrogen, then stored at –70°C for subsequent analyses. Extracts of *Drosophila* were prepared as described (Henikoff et al. 1986). Protein was quantified using a modified Bradford assay kit (Biorad). The Dam methylase reactions were performed in a 20 µl volume with 2 µg of unmethylated lambda DNA, 80 µM S-adenosylmethionine, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 5 mM β-mercaptoethanol. The *Drosophila* assays contained 10 µl extract and 2 µl Dam methylase storage buffer (New England Biolabs). The reactions for methylase assay of recombinants contained 10 µl protein extraction buffer and varying amounts of enzyme in 2 µl storage buffer. Reactions were incubated for 1 h at 37°C, followed by 15 min at 68°C. DNA was extracted using phenol and chloroform and precipitated with ethanol. *Dpn*I and *Dpn*II restriction digests were performed using 0.5 µg of lambda DNA. Following agarose gel electrophoresis, restriction digests of DNA incubated with *Drosophila* protein were compared with DNA incubated with various quantities of recombinant Dam methylase to allow an estimation of methylase activity in extracts.

Slot-blot analysis. Genomic DNA of *Drosophila melanogaster* was purified by the method of Bender et al. (1983) as modified by J. Hirsh, which involves lysis and multiple precipitations, including spermine. Digested DNA was denatured by addition of 0.1 vol. of 3 N NaOH, followed by incubation at 68°C for 30 min; it was then applied to a nylon membrane in a slot-blot apparatus at a load of two fly equivalents per slot. 150 ng of pBR322 DNA was included as a negative control. Membranes were probed with oligonucleotides end-labeled with [^{32}P]phosphate by T4 polynucleotide kinase. Hybridization was at 37°C for 4–16 h. After hybridization, filters were washed at room temperature in 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 0.05% sodium pyrophosphate and 0.1% SDS, then in 6 × SSC, 0.05% sodium pyrophosphate for 5 min each, then in the same solution at 10°C below T_m for 10 min. Filters were exposed to time-resolved fluorescent plates for processing using a phosphorimager (Molecular Dynamics), then stripped for reprobing.

Gel analysis. After endonuclease digestion, ten fly equivalents of purified genomic DNA from male flies was electrophoresed on 2.0% agarose gels and transferred to GeneScreen Plus membrane (New England Nuclear) for Southern analysis. Southern blots were sequentially hybridized to probes labeled with [^{32}P]nucleotides by random priming (Feinberg and Vogelstein 1983). The *Prat* probe was a 1.6 kb genomic fragment consisting of part of the coding region. The *rolled* probe was a full length cDNA (kindly provided by William Biggs).

Position-effect variegation (PEV) of P[lacW]. The X-ray mutagenesis screen that resulted in isolation of *T(2;3)Ch, P[lacW](61E)* has been described (Wines and Henikoff 1992). Crosses to generate *Y*-dosage lines and the staining of imaginal disks with anti-β-galactosidase antibody were performed as described (Wines and Henikoff 1992).

Results

Flies with Dam methylase develop normally

We expected that introduction of ~10^6 methyl moieties per genome (as expected for a four-base target sequence) into the major groove of DNA would be highly detrimental to the development of a complex eukaryote. Therefore, a two-component system was used in which P transposase-mediated transformants should be obtained even when expression of the inserted gene is lethal. A pDAM plasmid was constructed by subcloning the coding region of the *dam* gene into a vector that directs *S. cerevisiae GAL4*-dependent transcription of an insert in *D. melanogaster* (Brand and Perrimon 1993). To activate expression, a transformed line carrying the inserted gene is crossed to a strain carrying HS-*GAL4*, a transposon that provides GAL4 activator protein under heat shock inducible control. Embryos were injected (Spradling 1986) with pDAM, and ten independently transformed lines were isolated, of which Me1-Me4 were chosen for detailed characterization.

Table 1. Development times of methylase-bearing flies

	Females	Males
Me1/HS-*GAL4*	10.6±1.9	10.8±1.6
CyO/HS-*GAL4*	10.7±1.4	11.1±1.6
Me1/HS-*GAL4* (+heat shock)	10.6±1.6	10.9±1.6
CyO/HS-*GAL4* (+heat shock)	11.0±1.6	11.0±1.4
Me2/HS-*GAL4*	10.4±1.5	10.6±1.6
CyO/HS-*GAL4*	10.9±1.6	10.8±1.5
Me2/HS-*GAL4* (+heat shock)	10.2±1.2	10.5±1.1
CyO/HS-*GAL4* (+heat shock)	10.9±1.4	10.8±1.4

The number of days (mean±standard deviation) from egg-laying to eclosion at 25° C is shown

The viability of Me1 and Me2 was assessed by comparing the mean developmental time of progeny carrying both the pDAM transposon and HS-*GAL4* with siblings that lack pDAM, with or without heat shock. In all cases examined, the presence of either Me1 or Me2 was not associated with any visible phenotype, nor did it cause any detectable change in developmental timing from egg deposition until eclosion for either females or males (Table 1). For example, heat-shocked Me1/HS-*GAL4* flies developed in 10.7±1.6 days compared with 11.0±1.5 days for their *CyO*/HS-*GAL4* siblings.

Detection of methylase activity in fly extracts

To confirm that methylase was being expressed in flies, we assayed the enzyme directly in extracts (Table 2). For the Me1-4 lines, methylase activity was undetectable in the absence of HS-*GAL4*. Flies from the Me1, Me2 and Me4 lines grown continuously at 25°C and aged 3 days showed substantial methylase activity in the presence of the HS-*GAL4* activator, averaging ~10^5 active methylase molecules per cell. Such activation of expression probably results from leaky expression of HS-*GAL4*, as has been reported previously (Speicher et al. 1994). When flies were subjected to heat shock during development, the level of methylase activity increased markedly in all three lines. This increase likely reflects induction of GAL4 expression by heat shock, with the consequent upregulation of Dam methylase by GAL4. In the Me3 line, methylase activity was not detected even in the presence of the HS-*GAL4* element after heat shock, suggesting that the methylase gene in the Me3 line is defective.

Methylation of endogenous DNA

We also examined methylation in vivo by digesting genomic DNA from the methylase lines with restriction

Table 2. Dam methylase assays of *Drosophila* extracts

Genotype	Activity
Me1/*CyO*	–
Me1/HS-*GAL4*	++
Me1/HS-*GAL4* (+heat shock)	+++
Me2/*CyO*	–
Me2/HS-*GAL4*	+
Me2/HS-*GAL4* (+heat shock)	+++
Me3/*CyO*	–
Me3/HS-*GAL4*	–
Me3/HS-*GAL4* (+heat shock)	–
Me4/Me4	–
Me4/HS-*GAL4*	+
Me4/HS-*GAL4* (+heat shock)	++

Classes are: +++ (>20 units/mg protein), ++ (10–20 units/mg), + (5–10 units/mg), - (no detectable activity). One unit corresponds to the amount of activity found in ~1 ng of pure Dam methylase, and fully protects 1 μg of lambda DNA at 37° C in a 10 μl reaction mixture against cleavage by *Mbo*I restriction endonuclease (New England Biolabs) for 1 h

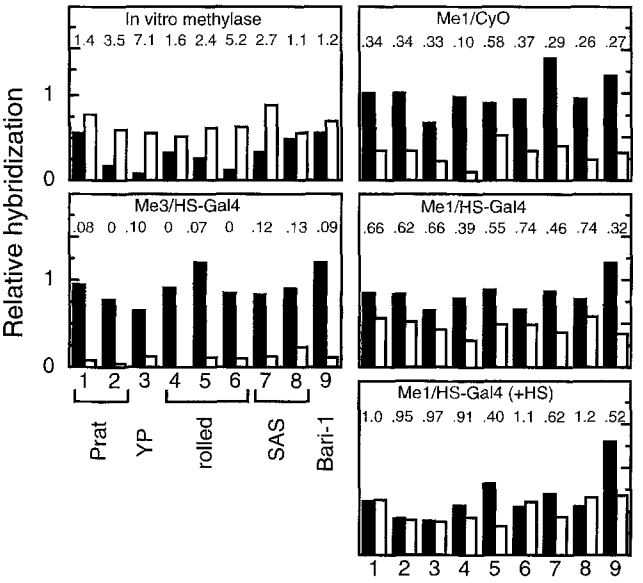


Fig. 1. Hybridization to genomic DNA on slot blots using oligonucleotide probes spanning GATC sites. To correct for loading differences between slots, a *Prat*-derived oligonucleotide that does not span a GATC site, 5′-GTTTACTTCGCCCGCGGAGAC-3′, was hybridized to each slot blot, and the resulting phosphorimager measurement for each slot was divided into each of the other measurements from the same slot. Nonspecific hybridization was corrected for by subtracting the signal in a slot containing pBR322 DNA. Relative hybridization refers to the ratio of the corrected signal for the indicated slot to that obtained for *Dpn*I digestion of unmethylated DNA present in a different slot. Relative hybridizations for *Dpn*I (*filled bars*) and *Dpn*II (*open bars*) are displayed. The ratio of *Dpn*II to *Dpn*I relative hybridizations (*numbers above bars*) should represent the ratio of dimethylated to unmethylated DNA, where any hemimethylated DNA contributes to the uncut fraction using either *Dpn*I or *Dpn*II. For a fully unmethylated site, *Dpn*I will fail to cut and so the hybridizable segment will be fully labeled (a tall filled bar), whereas *Dpn*II will cut fully and so the hybridizable segment will be unlabelled (no open bar). For a fully methylated site, the reverse situation is obtained, leading to no filled bar and a tall open bar. The normalization procedure introduces random errors that are difficult to quantify for single data points. However, consistent increases in measured levels of methylation with increasing methylase provide confirmation of accuracy. We attribute the single exceptional case, the apparent decrease in methylation at high levels of methylase for no. 5, to random error. The *Prat* oligonucleotide probes were derived from the 1.6 kb segment used for Southern analysis: 5′-GGCTTCCTGTCGATCGGTGCCAG-3′ (1); 5′-GGCCAGCTAGGATCAGGCATTTCA-3′ (2). The YP probe was derived from exon 1 of the *Yp2* gene: 5′-GGTCGCCGATCCAACTCC-3′ (3). The *rolled* probes were derived from the 5′ end, 5′-GGTACAGGATCTACGGAA-3′ (4); middle, 5′-TATTGCAGATCCCGAGCA-3′ (5); and 3′ end of the cDNA, 5′-GGCTTTAGATCTCCTTGG-3′(6). The SAS probes were from 898–921, 5′-TTAATCTACCTGATCCGAAAAACG-3′ (7); and from 954–975, 5′-GCTTTGTATGATCCAGACGGTT-3′ (8) based on published coordinates (Donnelly and Kiefer 1987). The *Bari-1* probe was 5′-GCAAATGAAGAGATCTTTATCAGTTG-3′ (9)

endonucleases that are specific for the GATC target site but are differentially sensitive to Dam methylation of the N^6 position of adenine in GATC (Brooks et al. 1983). *Dpn*I cleaves only dimethylated DNA and *Dpn*II cleaves only unmethylated DNA (Lacks and Greenberg 1977). To detect and quantify cleavage at a site we used an oligonucleotide probing method (Kladde and Simpson 1994). Each short radiolabeled probe spans a particular GATC site, such that hybrids can form only when the site is intact. Following hybridization to samples on a slot blot, the amount of uncut DNA is measured by phosphorimaging. Relative hybridization levels using these probes are shown (Fig. 1) for DNA digested with *Dpn*I (filled bars) versus DNA digested with *Dpn*II (open bars). When methylated DNA is present, *Dpn*I hybridization is reduced (filled bars lower) and *Dpn*II hybridization is increased (open bars higher), and *vice versa* for unmethylated DNA. Comparison of ratios for each probe allows estimation of the extent of dimethylation at each particular GATC site examined. A variety of oligonucleotide probes were chosen to assess what types of change would be detectable in this assay. *Prat*, which encodes phosphoribosylamidotransferase, is a housekeeping gene located in euchromatin that is expressed throughout development (Clark 1994). *Yp2*, which encodes a yolk protein, is a euchromatic locus active in the female but not in the male flies (Hovemann and Galler 1982) used in this experiment. *rolled*, which encodes the *Drosophila* ERK-A homolog, is a housekeeping gene located within the proximal heterochromatin of chromosomal arm *2R* that is expressed in both embryos and adult flies (Biggs and Zipursky 1992). *SAS* is a satellite-associated sequence that is repeated about 50 times (Donnelly and Kiefer 1986). The majority of the *SAS* copies should represent non-transcribed heterochromatic sequences. *Bari-1* is a transposon repeat present almost entirely in a single cluster of ~20 copies very near the centromere in the heterochromatin of *2R* (Caizzi et al. 1993).

To establish the limits of this oligonucleotide-based assay, we probed genomic DNA samples that were methylated by treatment in vitro (Fig. 1, upper left) and other samples from the Me3/HS-*GAL4* line (middle left) that lacked detectable methylase activity in extracts or on Southern blots (Fig. 2). For in vitro methylated DNA,

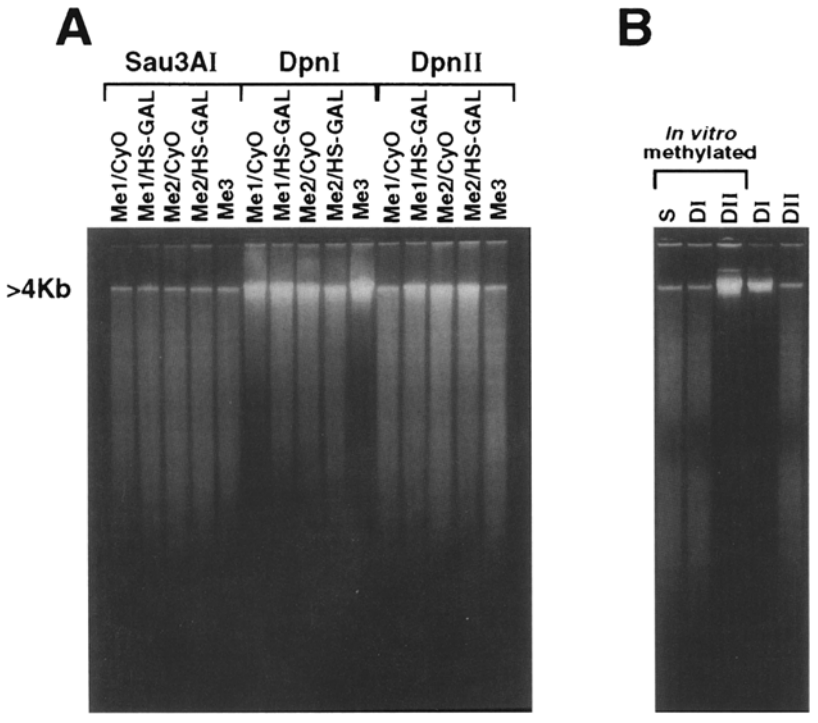
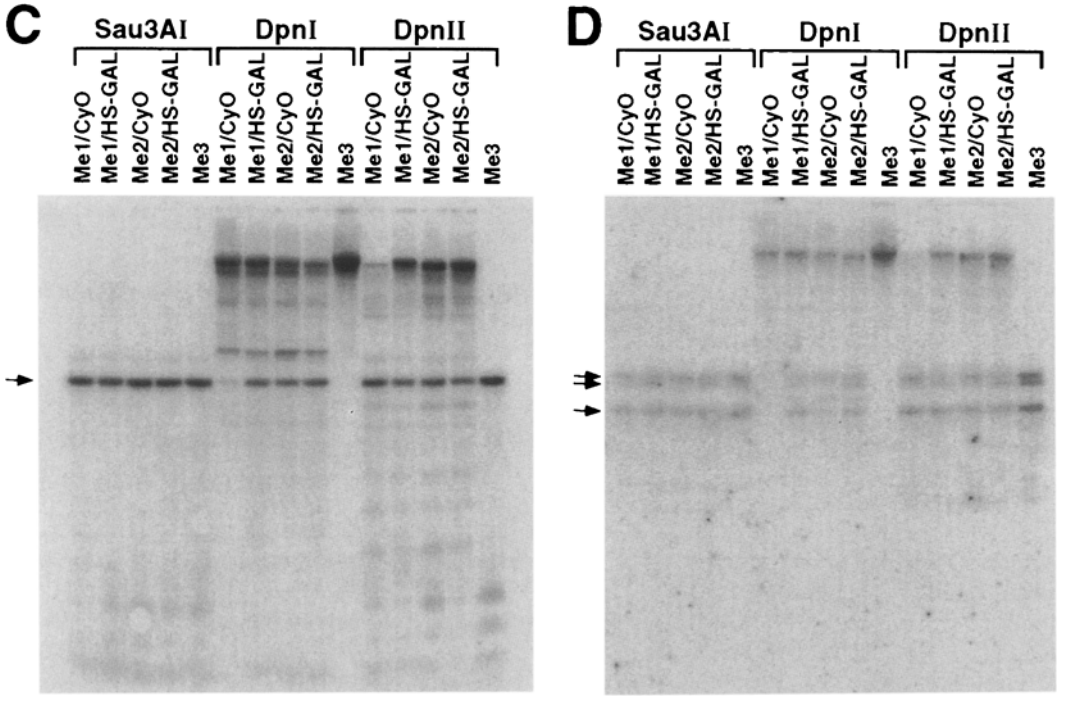


Fig. 2A–D. Detection of methylation in vivo by Southern analysis of genomic DNA. **A** *Sau*3AI, *Dpn*I and *Dpn*II digests of genomic DNA from the Me1, Me2 and Me3 lines electrophoresed on a 2% agarose gel. The zone of compression contains all DNA fragments greater than 4 kb in size. No heat shocks were administered to flies used for preparation of DNA. **B** In vitro methylation control. DNA from the HS-*GAL4* activator strain was methylated in vitro with recombinant Dam methylase (New England Biolabs), digested with *Sau*3AI (*S*), *Dpn*I (*DI*) and *Dpn*II (*DII*) and electrophoresed on a 2% agarose gel. Unmethylated DNA (*right*) from the same sample was digested in parallel. **C,D** Sequential hybridization of a Southern blot of the gel shown in **A** to probes from *Prat* (**C**) and *rolled* (**D**). *Arrows* point to higher molecular weight bands from complete digests



greater relative hybridization was seen for *Dpn*II-digested DNA than for *Dpn*I-digested DNA, as expected. However, with some probes, significant hybridization to *Dpn*I-digested DNA was detected, which might be due to the presence of tightly bound protein (Werner et al. 1984) that survived our standard lysis-spermine precipitation procedure for DNA purification (Bender et al. 1983). For Me3/HS-*GAL4* DNA, much greater relative hybridization was seen for *Dpn*I-digested DNA than for *Dpn*II-digested DNA, indicating that little or no methylation occurred in this line.

DNA samples from various Me1-bearing lines were also probed (Fig. 1, right). Although Me1/*CyO* flies lack detectable methylase in extracts, low to moderate levels of methylation were observed, evidently the result of very low expression. Methylation increased in the presence of HS-*GAL4* and further increased with heat-shock treatment, consistent with the results of methylase assays (Table 2). It is striking that in no case did the level of methylation increase to more than about 50%, as though about half of *Drosophila* DNA is refractory to methylation in vivo, even at high methylase concentrations. Sim-

ilar results were obtained with DNA from Me2-bearing lines (data not shown). An attractive explanation for this observation is that nucleosomes block Dam methylase from modifying half of the DNA, as is the case for phased nucleosomes in yeast (Kladde and Simpson 1994).

To address the possibility that partial methylation resulted from absence of methylase from a large fraction of cells, we examined methylation by electrophoresis in agarose gels. If nucleosomes are blocking methylation, a mixture of bands should result from full and partial digestion by either *Dpn*I or *Dpn*II; however, if DNA from some cells is not exposed to methylase, then partial digestion should not occur, since adjacent sites would be either fully digested or fully undigested. For both Me1 and Me2, partial digestion is the rule, as is evident for the bulk of DNA (Fig. 2A), as well as for *Prat* in the presence of high levels of methylase (Fig. 2C). Using a *rolled* probe, partial digestion bands are less obvious (Fig. 2D). Therefore we cannot rule out the possibility that contiguous sites in heterochromatin are relatively sequestered from methylase in a fraction of the cells. This uncertainty cannot be resolved using the quantitative slot-blot assay, because neighboring sites are examined independently.

These gel-based results also argue against the possibility that our analysis is complicated by hemi-methylation: hemi-methylation results in failure of both *Dpn*I and *Dpn*II but not Sau3AI to cleave. The intensities of bands produced by complete digestion with *Sau*3AI are no higher than expected for the sum of intensities for the corresponding *Dpn*I and *Dpn*II bands. This is a very sensitive test, because each of these bands is produced by two neighboring GATC sites and hemi-methylation at either one would lead to loss of material for both *Dpn*I and *Dpn*II relative to *Sau*3AI.

Site-specific differences in accessibility

Overall, no consistent differences were seen between euchromatic and heterochromatic sites when methylase was present at levels that could be detected in extracts. This was seen to be the case when methylation was assayed quantitatively in slot blot assays (Fig. 1) or qualitatively by gel electrophoresis (Fig. 2). For example, Me1/HS-*GAL4* flies subjected to heat-shock treatment showed ~50% methylation for all three euchromatic sites and three of the six heterochromatic sites examined on slot blots (Fig. 1, lower right). Similarly, the degree of methylation detected by a *Prat* probe on gels was similar to that detected by a *rolled* probe for both Me1 and Me2 in the presence of HS-*GAL4* (Fig. 2C,D). However, there were exceptions. For example, *rolled* sites at the 5′ end (Fig. 1, no. 4) and the 3′ end (no. 6) became more frequently methylated with increasing methylase activity, whereas a *rolled* site in between (no. 5) showed no increase in methylation from low to high levels of methylase. This could reflect an unusual chromatin structure of this site in most cells.

At levels of methylase below detection in extracts, more differences were seen between euchromatic and heterochromatic sites. For example, a higher level of partial methylation was detected by the *Prat* probe than by the *rolled* probe in the Me1 line (Fig. 2C,D). Site-specific differences between *Prat* and *rolled* were not seen in the Me2 line, which appeared to have a higher level of endogenous methylation overall in the absence of HS-*GAL4*.

Position-effect variegation appears to have little effect on overall accessibility

Site-to-site variations in the degree of methylation suggested that methylation differences might be detected between euchromatin and heterochromatin by examining a gene that is subject to PEV. In PEV, a gene is variably silenced when it is juxtaposed to heterochromatin by a rearrangement (reviewed by Henikoff 1990). PEV should permit examination of the same gene in its normal euchromatic state and in its rearranged heterochromatic state. To obtain a suitable example of PEV to assay methylation, we started with a third chromosomal line carrying a *P[lacW]* transposon inserted into 61E, which showed relatively uniform *lacZ* expression in a wide variety of cells throughout development (Wines and Henikoff 1992). PEV was induced by X-irradiation with selection for variegation of the mini-*white* reporter within the transposon. Cytological examination of one variegating line, designated *T(2;3)Ch* [T(2;3) 2het;61E], revealed a translocation placing second chromosome heterochromatin immediately proximal to the transposon. We confirmed that *T(2;3)Ch* is a *bona fide* example of PEV by the sensitivity of mini-*white* to PEV modifiers, such as dosage of the *Y* chromosome (Fig. 3A). Most importantly, *X/O* males had white eyes, indicating complete heterochromatic silencing of mini-*white*. Therefore, we assumed that changes in methylase accessibility resulting from formation of heterochromatin would be detectable by comparing these males with males from the parental line.

lacZ DNA was used to probe Southern blots containing DNA from flies heterozygous for either *P[lacW](61E)* or *T(2;3)Ch* and Me4, which expresses low levels of methylase (Table 2). Only minor differences were detected among the three tested genotypes: *X/Y, P[lacW](61E)/Me4; X/Y, T(2;3)Ch/Me4* and *X/O, T(2,3)Ch/Me4* (Fig. 4). Reduced accessibility to methylase is inferred from the pattern of *Dpn*I digestion: all four limit digest bands (arrows in Fig. 4) are visible only in *P[lacW](61E)* DNA, and not in either *X/Y; T(2;3)Ch* or *X/O; T(2;3)Ch*. However, no comparable accessibility differences are apparent among any of the tested genotypes in *Dpn*II-digested DNA, nor between *X/Y* and *X/O T(2;3)Ch* males. Moreover, slot blot analysis of two *lacZ* sites revealed no significant differences among any of the DNA samples displayed in Fig. 4, although the two sites differed from one another. For one site, the ratio of methylated to unmethylated DNA ranged from 0.33–0.40, whereas for the other site, the ratio ranged from 0.60–0.70 (data not shown). These results support the previous conclusion, based on comparing methylation between euchromatic and heterochromatic genes, that the heterochromatic state does not prevent methylase accessibility in vivo.

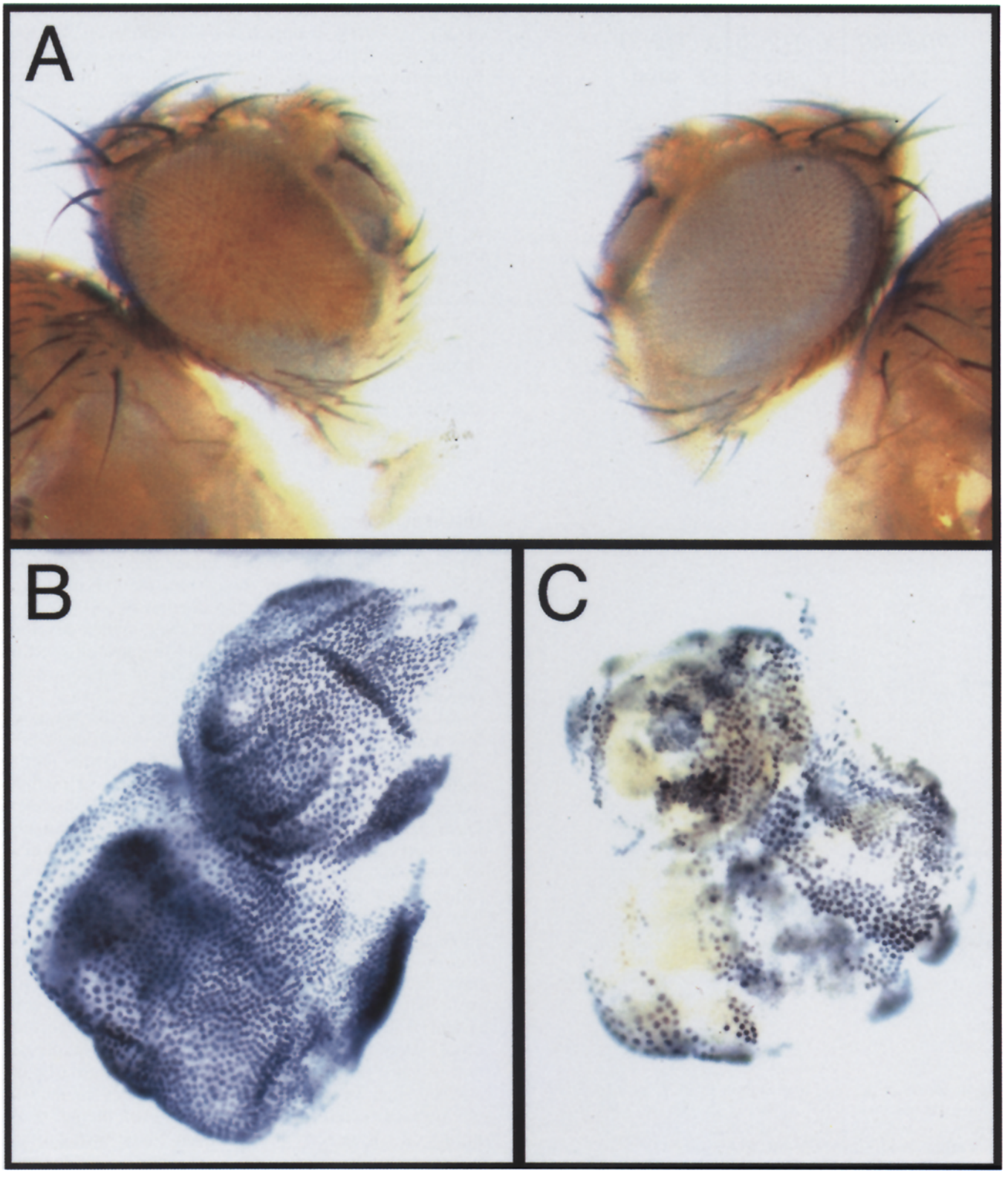


Fig. 3A–C. Position-effect variegation (PEV) occurs in developing tissue of *T(2;3)Ch* and is enhanced in *X/O* males. **A** Eye pigmentation of typical *X/Y* (*left*) and *X/O* (*right*) males. **B,C** β-galactosidase staining of an eye-antennal imaginal disk from an *X/Y* male (**B**), which is indistinguishable from a stained disc of the *P[lacW](61E)* parental line (not shown), and of a disk from an *X/O* male (**C**)

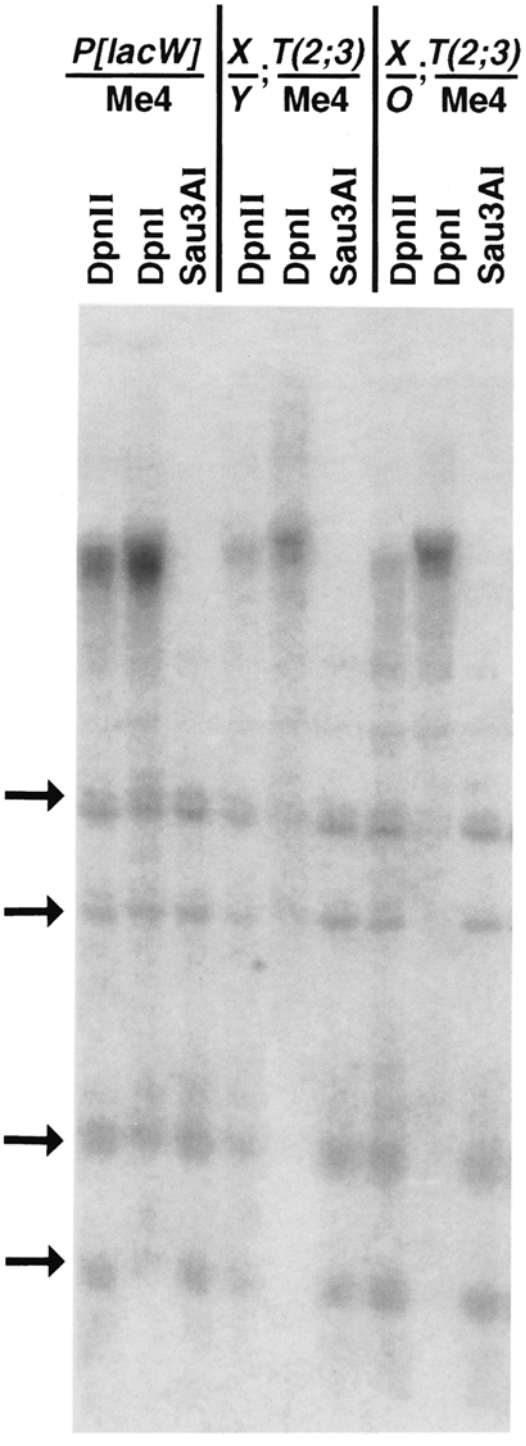


Fig. 4. Detection of *lacZ* methylation in PEV lines by Southern analysis of genomic DNA as in Fig. 2. Plasmid pMC1871 was used for probing *lacZ* (Casadaban et al. 1983). *Arrows* indicate limit digest bands

PEV silencing appears to change abruptly during development

The use of a PEV line with a *lacZ* reporter expressed throughout development permitted us to ascertain whether PEV of *white* in the adult eye reflects similar silencing in developing tissue. We were surprised to find that this is not the case. In eye-antennal imaginal disks of *X/Y; T(2;3)Ch* males, full *lacZ* expression was observed (Fig. 3B), rather than strong "pepper-and-salt" variegation as expected from the adult eye phenotype (Fig. 3A, left). In eye disks of *X/O; T(2;3)Ch* males, striking variegation was apparent (Fig. 3C), not complete inactivation (as in the eyes, see Fig. 3A, right). This quantitative difference is consistent with the possibility that these two different reporter genes are differentially sensitive to silencing by heterochromatin. However, the distribution also differed qualitatively, showing patchy rather than pepper-and-salt pigmentation, indicative of early clonal inactivation. Similar patchy expression had been observed both in adult eyes for mini-*white* and in disks for *lacZ* owing to clonal loss of the same *P[lacW]* insertion present on the *Dp(3;f)Th* unstable chromosome (Wines and Henikoff 1992). Such quantitative and qualitative differences occurring in the same tissue during development for adjacent reporter genes suggest that chromatin structure can change dynamically during development.

Discussion

We have shown that flies tolerate extensive artificial DNA modification, even though no natural modifications are known to occur. Introduction of ~10^6 methyl groups per genome does not affect the complex developmental program of *Drosophila*, and this has allowed us to probe chromatin structure in vivo in a noninvasive manner.

At high levels of *dam* methylase gene expression, we found a maximum of about 50% methylation at diverse sites. It would seem extremely unlikely that this partial methylation resulted from insufficient levels of methylase in cells. First, the concentration of active Dam methylase measured relative to its substrate in flies, estimated to be 1 molecule per <10 kb DNA, exceeded the concentration found naturally in rapidly dividing *E. coli* cells [1 molecule per 30 kb (Boye et al. 1992)]. Second, enzyme was assayed and methylation of endogenous DNA measured several days after the completion of cell proliferation during development, so that sufficient time should have been available for all accessible sites to become methylated. Third, 50% methylation of each site is consistent with the observation that a phased nucleosome blocks Dam methylase from DNA to a comparable extent in yeast (Kladde and Simpson 1994). Similarly, we attribute this limiting degree of refractivity to the nucleosomal structure of *Drosophila* DNA, although phasing might not be seen in DNA from whole flies because of tissue-specific differences.

Given that methylase levels were so high in cells for such long periods of time in the presence of HS-*GAL4*, it is surprising that detectable increases in the degree of methylation occurred with heat-shock treatments during development. This suggests that either the effective levels of methylase were much lower than measured in extracts, or that methylated bases were removed by DNA repair enzymes, or both. Effective levels may have been

low if methylation occurred only during replication. It is possible that during replication, euchromatin and heterochromatin are nearly equally accessible, so that differences are only seen when methylase is present at limiting levels.

At high levels of methylase we did not detect consistent differences between sites in euchromatin and sites in heterochromatin, suggesting that the highly condensed state of heterochromatin does not prevent access to a freely diffusible protein. This interpretation is in agreement with nuclease accessibility studies (Hayashi et al. 1990; Schlossherr et al. 1994; Locke 1994). However, at methylase levels below detection in extracts, overall accessibility of the heterochromatic *rolled* gene was slightly reduced relative to that of the euchromatic *Prat* gene, as was the case for *lacZ* subject to PEV relative to the same gene in the parental line. It is possible that low levels of methylase are necessary in order to detect higher order differences in chromatin structure, such as the difference between heterochromatin and euchromatin. We note that the refractivity of silenced telomeric DNA to methylase in yeast (Gottschling 1992) was observed using the full *E. coli dam* gene, which lacks a yeast promoter, and so would be expected to synthesize low levels of methylase.

The use of a novel PEV line for methylase assays, in which silencing can be observed during development, also allowed us to ascertain whether fully differentiated phenotypes accurately represent earlier developmental decisions. Unexpectedly, we observed striking differences in inactivation of marker genes before and after differentiation. It is formally possible that these differences reflect differences in the precise locations of two adjacent reporter genes, since one lies slightly closer to the heterochromatic breakpoint than the other and so might be more strongly affected. Even if that were the case, it is hard to understand how a difference in strength of silencing can account for observed differences in pattern of variegation. Patchy variegation in the developing disk should reflect a very early event, and pepper-and-salt variegation in the eye should reflect a very late event. This lack of correspondence suggests that a profound structural change in chromatin occurs during differentiation of the eye. Our finding also has general implications for interpretation of PEV phenotypes, since these might not accurately reflect the inheritance of chromatin structure during development.

A limitation of our study is that the use of whole adult flies might have obscured important differences in methylase accessibility in different tissue types. Such differences should be detected more readily using purified tissues as a source of genomic DNA. This approach becomes feasible given our direct demonstration that PEV occurs in undifferentiated diploid cells. For detection of chromatin structure in purified tissues, in situ methods seem most appealing, perhaps using the polymerase chain reaction (PCR) to distinguish cleaved from uncleaved sites. The availability of restriction endonucleases specific for both methylated and unmethylated DNA, and the lack of detrimental effects of methylated A bases in flies, make the Dam methylase system an attractive one for introduction into other complex organisms, including vertebrates.

Acknowledgements. We thank M.K. Raghuraman for the *dam* methylase gene, Linda Martin-Morris for performing embryo injections, Jeff Jackson for help with stock maintenance and Dan Gottschling for helpful discussions. This work was supported by the Howard Hughes Medical Institute. Support for P.B.T. and D.V.C. was obtained from an NIH grant to S.H.

References

Bender W, Spierer P, Hogness DS (1983) Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the *bithorax* complex in *Drosophila melanogaster*. J Mol Biol 168:17–33

Biggs WH, Zipursky SL (1992) Primary structure, expression and signal-dependent tyrosine phosphorylation of a *Drosophila* homolog of extracellular signal-regulated kinase. Proc Natl Acad Sci USA 89:6295–6299

Bird A (1992) The essentials of DNA methylation. Cell 70:5–8

Boye E, Marinus MG, Lobner-Olesen A (1992) Quantitation of Dam methyltransferase in *Escherichia coli*. J Bacteriol 174:1682–1685

Brand A, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415

Brooks JE, Blumenthal RM, Gingeras TR (1983) The isolation and characterization of the *Escherichia coli* DNA adenine methylase *(dam)* gene. Nucleic Acids Res 11:835–851

Caizzi R, Caggese C, Pimpinelli S (1993) Bari-1, a new transposon-like family in *Drosophila melanogaster* with a unique heterochromatic organization. Genetics 133:335–345

Casadaban MJ, Martinez-Arias A, Shapira MK, Chou J (1983) Beta-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol 100:293–308

Clark DV (1994) Molecular and genetic analyses of Drosophila *Prat*, which encodes the first enzyme of de novo purine biosynthesis. Genetics 136:547–557

Donnelly RJ, Kiefer BI (1986) DNA sequence adjacent to and specific for the 1.672 g/cm^3 satellite DNA in the *Drosophila* genome. Proc Natl Acad Sci USA 83:7172–7176

Donnelly RJ, Kiefer BI (1987) DNA sequence of a *Drosophila* satellite associated sequence. Nucleic Acids Res 15:10061

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Gottschling DE (1992) Telomere-proximal DNA in *Saccharomyces cerevisiae* is refractory to methyltransferase activity in vivo. Proc Natl Acad Sci USA 89:4062–4065

Hayashi S, Ruddell A, Sinclair D, Grigliatti T (1990) Chromosomal structure is altered by mutations that suppress or enhance position effect variegation. Chromosoma 99:391–400

Henikoff S (1990) Position-effect variegation after 60 years. Trends Genet 6:422–426

Henikoff S, Keene MA, Sloan JS, Bleskan J, Hards R, Patterson D (1986) Multiple purine pathway enzyme activities are encoded at a single genetic locus in *Drosophila*. Proc Natl Acad Sci USA 83:720–724

Hoekstra MF, Malone RE (1985) Expression of the *Escherichia coli dam* methylase gene in *Saccharomyces cerevisiae*: effect of in vitro adenine methylation on genetic recombination and mutation. Mol Cell Biol 5:610–618

Hovemann B, Galler R (1982) Vitellogenin in *Drosophila melanogaster*. A comparison of the YPI and YPII genes and their transcription products. Nucleic Acids Res 10:2261–2274

Jensen RE, Herskowitz I (1984) Directionality and regulation of casette substitution in yeast. Cold Spring Harbor Symp Quant Biol 49:97–104

Kladde MP, Simpson RT (1994) Positioned nucleosomes inhibit Dam methylase in vivo. Proc Natl Acad Sci USA 91:1361–1365

Lacks S, Greenberg B (1977) Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. J Mol Biol 114:153–168

Lindsley DL, Zimm GG (1992) The genome of *Drosophila melanogaster*. Academic Press, San Diego

Locke J (1994) Examination of DNA sequences undergoing chromatin conformation changes at a variegating breakpoint in *Drosophila melanogaster*. Genetica 92:33–41

Schlossherr J, Eggert H, Paro R, Cremer S, Jack RS (1994) Gene inactivation in *Drosophila* mediated by the *Polycomb* gene product or by position-effect variegation does not involve major changes in the accessiblity of the chromatin fibre. Mol Gen Genet 243:453–462

Singh J, Klar AJS (1992) Active genes in budding yeast display enhanced in vivo accessibility to foreign DNA methylases: a novel in vivo probe for chromatin structure of yeast. Genes Dev 6:186–196

Speicher SA, Thomas U, Hinz U, Knust E (1994) The *Serrate* locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. Development 120:535–544

Spradling AC (1986) P element-mediated transformation. In: Roberts DB (ed) Drosophila: *a practical approach*. IRL Press, Oxford, pp 175–197

Werner D, Chanpu S, Muller M, Spiess E, Plagens U (1984) Antibodies to the most tightly bound proteins in eukaryotic DNA. Formation of immuno-complexes with "nuclear matrix" components. Exp Cell Res 151:384–395

Wines DR, Henikoff S (1992) Somatic instability of a *Drosophila* chromosome. Genetics 131:683–691