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DNA representation of variegating heterochromatic P-element inserts in diploid and polytene tissues of *Drosophila melanogaster*

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Abstract. Position-effect variegation (PEV) is the mosaic expression of a euchromatic gene brought into juxtaposition with heterochromatin. Fourteen different transformed *Drosophila melanogaster* lines with variegating P-element inserts were used to examine the DNA levels of these transgenes. Insert sites include pericentric, telomeric and fourth chromosome regions. Southern blot analyses showed that the heterochromatic *hsp26* transgenes are underrepresented 1.3- to 33-fold in polytene tissue relative to the endogenous euchromatic *hsp26* gene. In contrast, the heterochromatic *hsp26* transgenes are present in approximately the same copy number as the endogenous euchromatic *hsp26* gene in diploid tissue. It appears unlikely that DNA loss could account for the lack of gene expression in diploid tissues seen with these examples of PEV.

Introduction

For over 60 years the phenomenon of position effect variegation (PEV) has been the subject of genetic and molecular research (for review see Henikoff 1990; Spradling and Karpen 1990). In 1930, H.J. Muller recovered flies of *Drosophila melanogaster* that possessed mottled eyes (referred to as "eversporting displacements") from an X-ray mutagenesis screen. In these flies the *white* gene, required for red eye pigmentation, is expressed in some, but not all, cells. Gene inactivation associated with PEV appears to be stable and clonally inherited. Today we know that PEV arises when a euchromatic gene is brought into juxtaposition with heterochromatin by a chromosomal rearrangement or P-element transposition event (for reviews see Lewis 1950; Spofford 1976; Weiler and Wakimoto 1995).

Heterochromatin was defined cytologically by Heitz (1928) as those regions of the genome that remain compact throughout the cell cycle, typically found around the centromeres. In *Drosophila* heterochromatin makes up the dense mass of the chromocenter, the region of physical association of all the centromeres in polytene chromosomes. *Drosophila* heterochromatin can be classified into two categories, α and β, based on morphological characteristics seen in polytene chromosomes of salivary glands (Heitz 1928). The α-heterochromatin is composed of highly repetitive satellite DNA sequences (Lohe et al. 1993) that are dramatically underrepresented relative to euchromatic sequences in polytene chromosomes, and form the central compact region of the chromocenter (Heitz 1928). The β-heterochromatin is composed of non-satellite repetitive sequences (for review see Miklos and Cotsell 1990) that are replicated during formation of polytene chromosomes and make up the bulk of the visible chromocenter (Heitz 1928; Traverse and Pardue 1989). In *Drosophila*, the small fourth chromosome is mostly heterochromatic and contains blocks of the same repetitive elements found in β-heterochromatin (for reviews see Hochman 1973; Miklos and Cotsell 1990). Rearrangements and P-element transpositions involving the fourth chromosome induce PEV of euchromatic genes (for review see Spofford 1976; Wallrath and Elgin 1995).

Cytologically, *Drosophila* telomeres do not appear to be heterochromatic (Gall et al. 1971; Pimpinelli et al. 1976) but their affect on gene expression suggests that they may be considered as such. Telomeres in *Drosophila* (Hazelrigg et al. 1984; Karpen and Spradling 1992; Wallrath and Elgin 1995), *Saccharomyces cerevisiae* (Renauld et al. 1993; Kyrion et al. 1993) and *Schizosaccharomyces pombe* (Nimmo et al. 1994) bring about gene silencing similar to that caused by pericentric heterochromatin. Repetitive DNA sequences found at telomeres in *Drosophila* are also found in centric heterochromatin and along the *Y* chromosome (Traverse and

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Pardue 1989; Valgeirsdóttir et al. 1990; Danilevskaya et al. 1993; Pardue 1995). For the above reasons we will consider *Drosophila* telomeres as heterochromatic in the following discussion.

Three types of models, not mutually exclusive, have been proposed to explain the loss of gene expression associated with PEV: (1) somatic DNA elimination; (2) alterations in chromatin packaging, and (3) nuclear compartmentalization. The DNA elimination model was first suggested by Schultz (1936) and more recently by Karpen and Spradling (1990) and Spradling (1993). According to this theory, the loss of gene expression is due to the genomic instability of heterochromatic DNA seen in the reduction in heterochromatin in polytene nuclei. The second model was originally based on cytological observations. In polytene chromosomes, the normally banded euchromatic regions may take on the appearance of a dense meshwork of chromatin fibers when juxtaposed to heterochromatin (Ananiev and Gvozdev 1974). This cytological change correlates with the inactivation of genes near the breakpoint (Zhimulev et al. 1986) and with an association with heterochromatin protein 1 (HP1) (Belyaeva et al. 1993). Alterations in cytology have been postulated to reflect structural changes in DNA compaction that lead to gene inactivation (Ananiev and Gvozdev 1974; Zhimulev et al. 1988). More recently, PEV has been shown to correlate with an alteration in chromatin structure at the nucleosomal level (Wallrath and Elgin 1995). Finally, the nuclear compartmentalization model suggests that the loss of gene expression associated with PEV is due to misplacement of the variegating gene(s) into a compartment within the nucleus that has inappropriate concentrations of necessary transcription components (Wakimoto and Hearn 1990; Karpen 1994). This model has been supported by observations on heterochromatic genes, that variegate when next to a breakpoint in the distal euchromatin of chromosomes *2* and *3* (Wakimoto and Hearn 1990)

In this report, we address the issue of DNA elimination as a mechanism for the loss of gene expression associated with PEV. We examine P-element inserts at 14 different variegating sites throughout the *Drosophila* genome (5 pericentric inserts, 4 telomeric inserts and 5 inserts at different sites along the fourth chromosome) for evidence of DNA elimination. We find no evidence for significant DNA elimination in diploid tissue. In contrast, we do find underrepresentation of heterochromatic transgenes in polytene tissue. Coupled with earlier observations on gene expression from such PEV lines (Wallrath and Elgin 1995), the data indicate that DNA loss does not correlate with the reduction in gene expression associated with PEV at pericentric and fourth chromosome sites.

Materials and methods

Fly stocks. All fly stocks used for the isolation of heads and salivary glands were raised on standard cornmeal/sucrose medium (Shaffer et al. 1994) at 23° C. All stocks were made homozygous for the particular P-element insert prior to use. For Fig. 2, flies were raised at 25° C and photographed 3–5 days posteclosion.

DNA isolation. DNA was isolated from salivary glands according to Di Franco et al. (1989) with minor modifications. Salivary glands were hand-dissected from 40–50 larvae in 0.8% NaCl and placed in a 1.5 ml microcentrifuge tube containing 200 µl of 0.8 M NaCl. Salivary glands were pelleted by centrifugation in a microcentrifuge and frozen at –20° C for later use. The pellet was resuspended in 200 µl of a solution containing 10 mM TRIS-HCl, pH 7.5, 1 mM EDTA and 0.1 M NaCl. Then 100 µl of 4 M NaCl, 100 µl of 1% SDS and 2 µl of Proteinase K (20 mg/ml) were added to the salivary gland suspension. The mixture was vortexed, placed at 50° C for 1 h and then cooled to 37° C. The mixture was treated once with 400 µl of phenol, once with 400 µl of phenol:chloroform:isoamyl alcohol (24:24:1), once with 400 µl of chloroform:isoamyl alcohol (24:1) and finally once with 400 µl of water-saturated ether. The purified DNA was precipitated with 800 µl of 95% ethanol at –20° C overnight. The DNA was pelleted by centrifugation for 20 min in a microcentrifuge at 4° C. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 20 µl of TE buffer (Sambrook et al. 1989). The DNA was then dialyzed against 40 ml of TE using a dialysis filter (Millipore, VMWP 013 00) for 30 min at room temperature. We found that without dialysis approximately 40% of the DNA samples did not digest to completion, even in the presence of excess restriction enzyme.

To obtain genomic DNA from adult heads, approximately 200–300 flies were collected in a 50 ml conical tube and the tube was submerged in liquid nitrogen. The tube was then pounded on the bench top many times (to disengage the heads from the bodies) and then emptied onto a no. 35 sieve (numerical assignment based on US standards; Fisher Scientific), which allowed the legs, wings and small body parts to filter through. The remaining material was sifted through a no. 30 sieve, which allowed the heads to pass through and the body parts to remain. Fifty heads were collected with a wet paintbrush and placed in a 1.5 ml microcentrifuge tube containing 200 µl of grinding buffer (Bender et al. 1983). The heads were ground at room temperature with a homogenizer and the suspension then incubated at 65° C for 30 min. Thirty microliters of 8 M potassium acetate was added to each tube; after incubation at 0° C for 30 min, the tube was spun in a microcentrifuge at 4° C for 10 min and the supernatant was transferred to a new microcentrifuge tube. The centrifugation was repeated, the supernatant was transferred to a new tube, and 250 µl of ethanol was added to each tube. The DNA was precipitated at room temperature for 5 min. The tubes were spun in a microcentrifuge at 4° C for 10 min. The pellet was washed twice with 100 µl of 70% ethanol, vacuum dried and dissolved in 20 µl of TE. The DNA was dialyzed against TE as described above.

Southern blotting. DNA isolated from 40–50 salivary glands or 50 heads was digested with 60 U of EcoRI (New England Biolabs) for 5 h at 37° C. The DNA was size separated on a 1% agarose TAE gel (Sambrook et al. 1989), denatured and transferred to a positively charged nylon membrane (Boehringer Mannheim) by capillary action using 10×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate). After transfer, the membrane was baked at 80° C under vacuum for 1 h. For membranes containing DNA isolated from heads, prehybridization and hybridization were performed according to Wallrath and Elgin (1995). The probe used was the EcoRI-PstI fragment from plasmid p88R6 (a subclone at the 5′ region of the *hsp26* gene) labeled with digoxigenin-dUTP according to instructions provided with the Boehringer Mannheim Genius System. Hybridization products were detected using the nonradioactive chemiluminescent alkaline phosphatase system (Boehringer Mannheim). We found that the amount of salivary gland DNA extracted from 40–50 glands was insufficient for nonradioactive detection on Southern blots. Therefore membranes containing DNA from salivary glands were prehybridized and hybridized according to Lu et al. (1992) and probed with the EcoRI-PstI fragment radioactively labeled by random priming (Feinberg and Vogelstein 1984) in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. The

membranes were exposed to preflashed X-OMAT film (Kodak). The autoradiographs and lumigraphs were scanned using a scanning densitometer (Molecular Dynamics). Two independent trials using the same lines showed average variations of ±0.05 for the ratio of transgene DNA to endogenous gene DNA.

In situ hybridization to polytene chromosomes. In situ hybridization was performed as described by Wallrath and Elgin (1995). The preparations of the fourth chromosome shown in Fig. 2 were made using Generation One, a mechanical chromosome-squashing apparatus (General Valve Corp., Fairfield, N.J.). The probe used was the entire P element containing the *hsp26-pt-T* and *hsp70-white* transgenes (Wallrath and Elgin 1995) labeled by nick translation in the presence of biotin-16-dUTP. Streptavidin-horseradish peroxidase complex (ENZO) and 3′–3′-diaminobenzidine (Sigma) were used for detection of the hybridization signal.

Results

Recovery of PEV lines

Seven of the PEV lines used here were recovered in a screen previously reported (Wallrath and Elgin 1995); seven additional lines were recovered in a continuation of that screen. The screen involves jumping a P-element with a marked copy of *hsp26* as a test gene, and *hsp70-white* as a reporter gene (Fig. 1), to various sites throughout the genome using *Δ2–3* as a source of transposase (Robertson et al. 1988). Flies exhibiting variegation of the *hsp70-white* gene are selected for further study. Together, these lines represent 14 independently isolated insertions in regions that bring about PEV: 5 pericentric insertions, 4 telomeric insertions and 5 inserts distributed along the length of the fourth chromosome. Flies homozgous for a pericentric or fourth chromosome insert are shown in Fig. 2. Telomeric inserts showed PEV of *hsp70-white* when hemizygous, but a wild-type or nearly wild-type phenotype when homozygous (approximately 80%–100% wild-type pigmentation) (Wallrath and Elgin 1995). There is little variation in eye phenotype among individuals of a given line.

In situ hybridization to polytene chromosomes of third instar larvae (Fig. 3) shows that the transgenes are inserted at pericentric locations on the right arm of chromosome 2 and on the *X* chromosome for lines 39C-2 and 118E-25, respectively. The pericentric *X*-linked insert line 118E-25 shows an interesting phenotype. Males exhibit wild-type levels of expression of *hsp70-white*, while females clearly show variegating expression (Fig. 2). Sex-linked differences in expression of the *hsp70-white* transgene are also observed with several *X*-linked

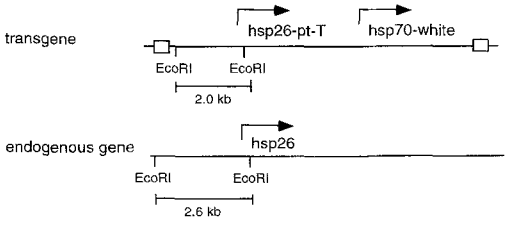

Fig. 1. Schematic diagram of the P-element construct and the endogenous *hsp26* gene. *Boxes* denote the inverted repeat of the P-element. *Arrows* denote the transcription start sites. The EcoRI sites used for Southern analysis are shown

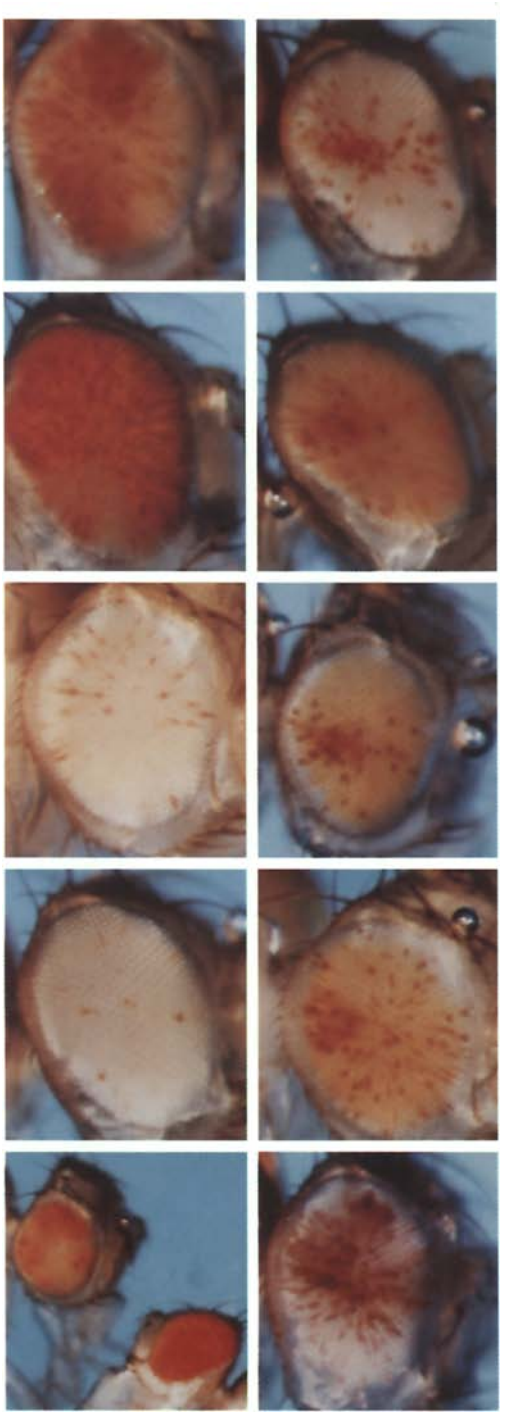

Fig. 2. Eye phenotype of lines with *hsp70-white* variegating inserts. Pericentric insertion lines are shown in the *left column* (*top to bottom*): 39C-2 (*2R* insert); 39C-3 (*2L* insert); 39C-4 (*2L* insert); 118E-12 (*3R* insert); 118E-25 (*X*-chromosome insert; female on left, male on right). Fourth chromosome insert lines are shown in the *right column* (*top to bottom*): 118E-10 (insert at cytological position 101); 118E-3 (insert at 102A-B); 39C-12 (insert at 102D); 39D-34 (insert at 102E-F); 118E-15 (insert at 102F)

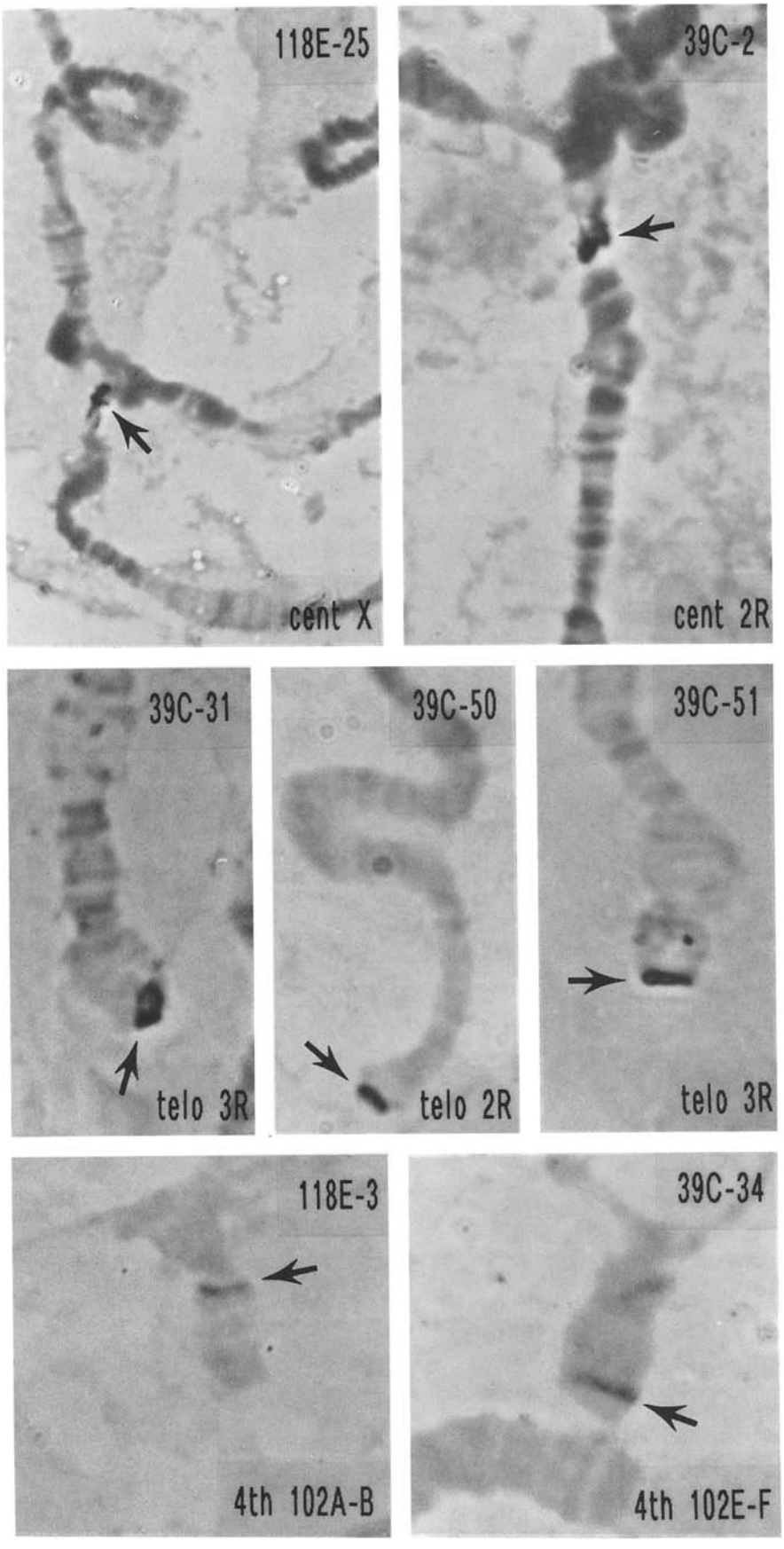

Fig. 3. In situ hybridization to polytene chromosomes of salivary glands from stocks showing position effect variegation of the *hsp70-white* transgene. Line designations are shown in the *upper right* and location of inserts in the *lower right* of each photograph. Lines 118E-25 and 39C-2 have pericentric insertions on the *X* and *2R*, respectively. Lines 39C-31, 39C-50 and 39C-51 have inserts near the telomeres of *3R*, *2R* and *3R* respectively. Lines 118E-3 and 39C-34 have inserts in the medial region of the fourth chromosome, at cytological positions 102A-B and 102E-F, respectively. The probe used was the P-element plasmid containing the *hsp26-pt-T* and *hsp-70-white* genes (Wallrath and Elgin 1995). *Arrows* show the sites of probe hybridization to the *hsp26-pt-T* transgene

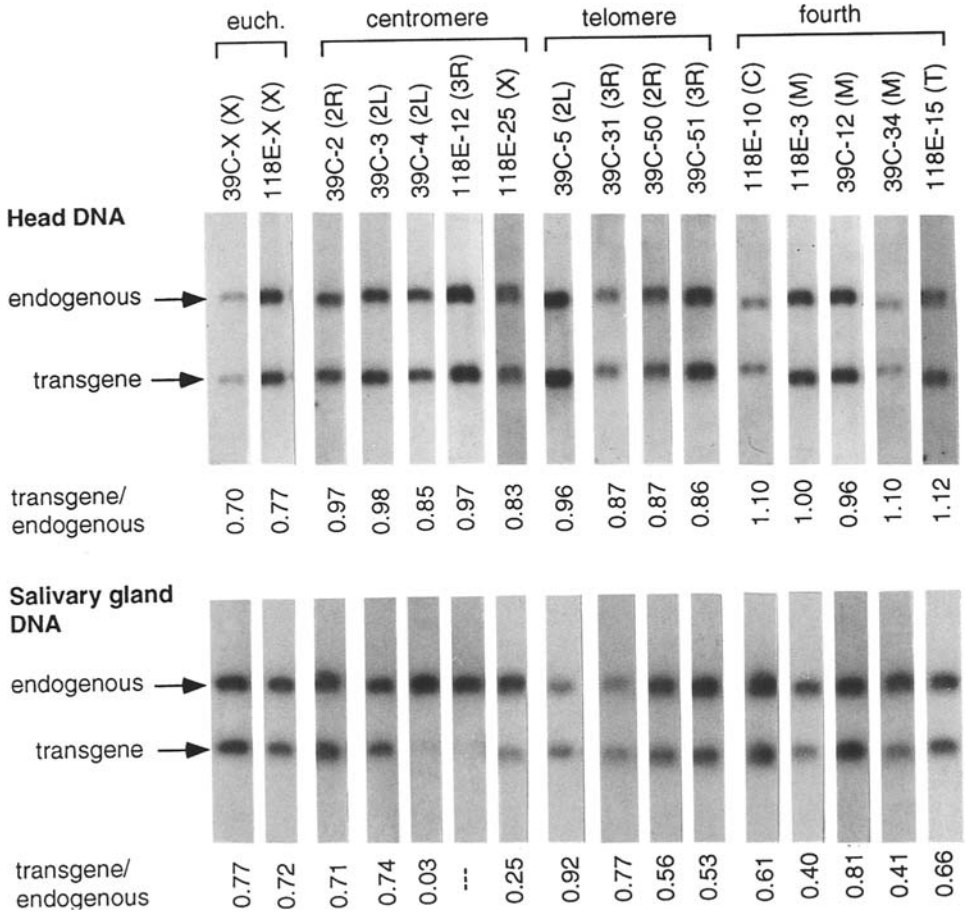

Fig. 4. Insertions of *hsp26-pt-T* are underrepresented in polytene tissue, but not in diploid tissue. DNA samples isolated from adult heads (diploid tissue) and larval salivary glands (polytene tissue) were cleaved with EcoRI and used for Southern blot analyses. A 5′ fragment of the *hsp26* gene, which hybridizes equally well to both the endogenous *hsp26* gene and the *hsp26-pt-T* transgene, was used as a probe (see Materials and methods). The line used is shown above each lane and the chromosomal location of the P-element insert is given in *parentheses*. The signal intensity of the *hsp26-pt-T* transgene relative to that of the endogenous *hsp26* gene is shown below each lane. Abbreviations: *X* chromosome, *C* centromere, *T* telomere, *M* medial region of the fourth chromosome

recovered in a similar screen (L.L. Wallrath, S.C.R. Elgin, unpublished results); this has not been seen with any of the 56 autosomal inserts showing PEV obtained from these screens. A sex-linked difference in expression of variegation is also seen for the *X*-linked *ecs* gene when juxtaposed to autosomal centric heterochromatin (Zhimulev et al. 1988; Belyaeva and Zhimulev 1991). These results suggest that the hyperactivation of *X*-linked genes in the male achieved by dosage compensation (reviewed by Baker et al. 1994) is functioning even in heterochromatic domains. Perhaps proteins involved in transcription and dosage compensation can compete against the heterochromatin proteins involved in packaging at these sites. There is a precedent for in vivo competition similar to that suggested here in reports on silencing at yeast telomeric sites (Renauld et al. 1993; Apariclo and Gottschling 1994).

Line 39C-50 has an insert near the telomere of *2R* and lines 39C-31 and 39C-51 have inserts near the telomere of *3R*. Lines 118E-3 and 39C-34 have inserts on the fourth chromosome, in cytological regions 102A-B and 102E-F, respectively. In situ hybridization results for the remainder of the lines used here are shown in Wallrath and Elgin (1995). In general, transgenes that were severely underrepresented in polytene tissue (see Southern analysis described below) were more difficult to detect by in situ hybridization to polytene chromosomes (e.g., line 118E-12; Wallrath and Elgin 1995) than lines showing representation similar to that of the endogenous euchromatic *hsp26* gene.

Heterochromatic hsp26 transgenes are present at comparable levels to the euchromatic hsp26 gene in diploid tissue

To examine whether DNA loss could account for the loss of gene expression at variegating sites we compared the amount of *hsp26-pt-T* transgene DNA to that of the endogenous *hsp26* gene in diploid tissue. Total genomic DNA was isolated from heads of adults for each of the transformed lines. The head contains primarily diploid cells, including those of the eyes, where the *white* PEV phenotype is observed. An EcoRI digest of the DNA yields a 2.6 kb fragment from the endogenous *hsp26* gene and a 2.0 kb fragment from the *hsp26-pt-T* transgene (Fig. 1); both fragments hybridize to an *hsp26* probe representing the 5′ portion of the gene (see Materials and methods). Densitometric scanning of the Southern blots showed that the ratio of transgene DNA to endogenous *hsp26* gene DNA in the euchromatic insert lines 39C-X and 118E-X was 0.70 and 0.77, respectively

(Fig. 4). This is close to the expected value of 0.75 (anticipated because lines 39C-X and 118E-X have *X*-linked inserts, present in a single copy in males). Note that the adults used for head isolation were not sexed (see Materials and methods). The ratio of transgene to endogenous gene was close to 1.00 for all PEV lines, indicating no significant DNA loss in diploid tissue. While the values obtained range from 0.83 to 1.12, there is no correlation between the deviation of the value obtained from 1.0, the extent of pigmentation in the eye, and the level of heat shock-induced expression of the *hsp26-pt-T* transgene (Wallrath and Elgin 1995), thus we attribute this range to random error. It should be pointed out that the white phenotype is scored for a small population of the cells; the DNA measurements cannot be made on a cell by cell basis, but are made for the tissue. A dramatic change in copy number of the transgene in a small number of cells could be missed. However, we note that in the cases studied there is a good correlation between the relative level of eye pigmentation and the inducible level of *hsp26-pt-T* expression for all pericentric and fourth chromosome inserts (Wallrath and Elgin 1995). Since heat shock genes are inducible in almost all cell types, we infer that the PEV observed here is likely to be operative in the bulk of the cell types in the head.

Heterochromatic hsp26 transgenes are underrepresented in polytene tissue

To examine the relative copy number of the *hsp26-pt-T* transgene in polytene tissue, total genomic DNA was isolated from salivary glands of third instar larvae. The two euchromatic *X*-linked insert lines, 39C-X and 118E-X, showed ratios of transgene DNA to endogenous *hsp26* DNA of 0.77 and 0.72, respectively (Fig. 4). Again, this value is close to the expected value of 0.75. Pericentric insertions showed a range of underrepresentation. For line 118E-12, signal from the *hsp26-pt-T* transgene was not detectable above background, even though signal from the endogenous gene was quite evident (Fig. 4). In line 39C-4, the transgene was 33-fold underrepresented compared with the endogenous gene. The *hsp26-pt-T* transgenes of lines 39C-2 and 39C-3 are approximately 1.4-fold underrepresented compared with the euchromatic *hsp26* gene. Both telomeric and fourth chromosome inserts showed underrepresentation ranging from 1.4- to 2.4-fold compared with the euchromatic gene. The exception is telomeric insert line 39C-5, which has a level of representation close to that of the euchromatic *hsp26* gene.

Discussion

Single P-element inserts in heterochromatin have allowed us to examine the replication state of different heterochromatic domains in both polytene and diploid tissues. While previous studies have focused on the representation of an allele at one variegating breakpoint (Henikoff 1981; Rushlow et al. 1984; Kornher and Kauffman 1986; Hayashi et al. 1990), the approach taken here allows comparison of the same transgene at a variety of heterochromatic sites. We have assayed multiple inserts for each of the three classes of heterochromatic regions causing variegation (pericentric, telomeric and fourth chromosome) to avoid any peculiarities that might be associated with a particular insertion site. We have used the variegating heterochromatic insert lines to examine transgene representation both in diploid cells of the head and in polytene chromosomes of the salivary gland.

Genomic instability as a possible mechanism for PEV

DNA elimination is seen in a variety of organisms as diverse as the horse parasite *Parascaris*, the Japanese hagfish *Eptatretus*, and the orchid *Dendrobium* (Nagl 1983; for review see Tobler et al. 1992; Kubota et al. 1993). In most cases, satellite DNAs found in heterochromatin are eliminated from somatic cells and retained in germ cells (Meyer and Lipps 1981; for a review see Tobler et al. 1992). The phenomenon is not limited to repetitious sequences, however; a ribosomal protein gene if *Ascaris* was recently shown to be eliminated from somatic cells during the process of chromatin diminution (discussed in Tobler et al. 1992). DNA elimination might then serve as a mechanisms of gene regulation. Thus, it is logical to suggest that the loss of gene expression seen with PEV could be accounted for by DNA elimination. Chromosomal instability in *Drosophila* has been reported by Spradling (1993). Nonrandom fragmentation of the *Dp1187* minichromosome has been seen in the ovary; production of chromosomal fragments there correlates with enhanced PEV of y^+ in the bristles and cuticle, suggesting loss of the y^+ DNA. Some of the truncated chromosomes, however, retained the telomeric y^+ allele, suggesting elimination from internal sites.

Several studies have been reported of variegating loci at a heterochromatic breakpoint in which DNA elimination is not detected. Hayashi et al. (1990) noted no change in copy number of a variegating *white* allele relative to a euchromatic gene in DNA from whole adults (primarily diploid cells). In a study of transgene inserts. Zhang and Spradling (1995) examined nine ry^+ inserts on the *Y* chromosome and eight *ry* inserts in centric heterochromatin and found no loss relative to the endogenous *ry* gene in DNA from whole adults. We do not find a significant reduction in *hsp26-pt-T* copy number relative to the endogenous *hsp26* gene in DNA isolated from heads of the variegated lines (Fig. 4). Based on the dramatic reduction in heat shock inducibility of *hsp26-pt-T* for several of these lines (e.g. to 4% of the euchromatic transgene value for line 118E-12; Wallrath and Elgin 1995), we would have expected to see significant loss of DNA if DNA elimination were the basis for this reduced expression.

PEV in relation to polytenization

Heterochromatin is known to be underrepresented in polytene tissues, including the salivary glands (Rudkin

1969). The reason for this underrepresentation of repetitious sequences in unclear. Several investigators have argued for underreplication as a cause of PEV (Roberts 1972; Ananiev and Gvozdev 1974). Laird (1973) hypothesized that if this were the case, stalled replication forks would be expected at heterochromatin/euchromatin junctions. However, Glaser et al. (1992) found no evidence of such structures at the euchromatic/heterochromatic junction of the minichromosome *Dp1187*, which contains mostly centric and telomeric regions of the *X* chromosome.

We find that most of the heterochromatic *hsp26-pt-T* transgenes are underrepresented relative to the endogenous *hsp26* gene in polytene tissue. Interestingly, there is a correlation between the amount of representation of the *hsp26-pt-T* transgene in polytene tissue and the amount of *white* expression in the eyes of the flies for pericentric insert lines, but not for fourth chromosome or telomeric insert lines. Lines 118E-12 and 39C-4 show less than 10% wild-type eye pigmentation (assessed by visual inspection) and have the lowest representation in polytene tissue: a 33-fold or greater reduction compared with the euchromatic *hsp26* gene (Figs. 2 and 4). Lines 39C-2 and 39C-3 show approximately 60%–80% *white* expression in the eye and exhibit only a 1.4-fold reduction in copy number relative to the endogenous *hsp26* gene.

These results showing underrepresentation of heterochromatic pericentric insertions in polytene cells are in contrast to those recently reported for a set of ry^+ P-element inserts (Zhang and Spradling 1995). Eight of these autosomal pericentric inserts showed representation equal to that of the endogenous *ry* gene in DNA isolated from salivary glands of third instar larvae. The authors suggest that full representation is due to the inserts being located in domains that become polytenized and loop out to form β-heterochromatin. This is in accordance with similar suggestions put forth by Traverse and Pardue (1989) on the formation of β-heterochromatin. The inserts described in this study might reside in regions that are underpolytenized and contribute to the chromocenter. However, in situ hybridization shows signals that are clearly not directly associated with the regions corresponding with α-heterochromatin (Fig. 3; Wallrath and Elgin 1995, Fig. 3). Many of the pericentric inserts gave a hybridization signal of irregular shape. Frequently triangular-shaped hybridization patterns and patterns showing two strands entering the chromocenter [similar to those reported in Zhang and Spradling (1995)] were seen (Fig. 3 and data not shown). It is not clear whether these unusual patterns of hybridization reflect underlying physical structures of heterochromatin.

Both differences and no differences have been reported for the DNA copy number of a variegating gene relative to a nonvariegating allele in polytene tissue. As examples, Henikoff (1981) found no difference in the copy number of the 87C heat shock locus in variegating and nonvariegating rearrangements, and Rushlow et al. (1984) found no difference in copy number between variegating and nonvariegating ry^+ alleles in the fat body and Malpighian tubules (both polytene tissues). In addition, Hayashi et al. (1990) found no difference in copy number of a variegating *white* allele and a euchromatic gene in salivary gland DNA. In contrast to these studies, a reduction in copy number has been seen for several variegating genes and chromosomal regions (Ananiev and Gvozdev 1974; Hartmann-Goldstein and Cowell 1976; Kornher and Kaufman 1986; Umbetova et al. 1991). Most notable are two examples from Spradling and his colleagues. First, a 39-fold underrepresentation in salivary gland DNA was found in the region of a variegating y^+ gene on minichromosome *Dp1187* in *XO* males (Karpen and Spradling 1992). Second, Zhang and Spradling (1995) reported a greater than 20-fold underrepresentation in polytene DNA of ry^+ inserts at nine locations on the *Y* chromosome.

Some of the discrepancy between findings may result from the use of different methodologies to determine DNA copy number. Feulgen staining (Hartmann-Goldstein and Cowell 1976), pulse labeling (Ananiev and Gvozdev 1974), in situ hybridization (Henikoff 1981) and Southern blotting (Rushlow 1984; Kornher and Kauffman 1986; Karpen and Sprading 1990; Zhang and Spradling 1995; this study) have all been used to measure the DNA content in chromosomal regions containing variegating genes. Henikoff (1981) found that the state of compaction of a chromosomal region, puffed or not puffed in polytene chromosomes, accounted for some variability in hybridization of a probe to that region. Efficiency in DNA transfer can be a problem with Southern blot analyses (discussed in Kornher and Kauffman 1986; Glaser and Spradling 1994). For this reason, we designed our experiments to compare relatively similar-sized, small restriction fragments that contain only sequences shared between the gene and transgene of interest.

Discrepancies might exist because the rearrangements and P-element insertions involve different domains within heterochromatin. Genetic evidence supports the notion that heterochromatin consists of domains of different sequences and chromosomal proteins that have different effects in causing variegation (Bishop 1992; Howe et al. 1995; Wallrath and Elgin 1995). For example, changes in the dosage of HP1 suppress centromeric and fourth chromosome PEV, but not telomeric PEV (Wallrath and Elgin 1995). The amount of surrounding heterochromatin may also influence the degree of representation of a variegating allele. Rearrangements often involve the juxtaposition of large blocks of euchromatin with large blocks of heterochromatin, whereas relatively small (10 kb) P-element inserts may be embedded within large regions of heterochromatin at great distances from euchromatin.

Because telomeres share some properties with centromeric heterochromatin, many researchers have wondered whether these are underrepresented in polytene chromosomes (for review see Pardue 1995). In the past it has been difficult to assess the copy number of telomeric DNA by Southern blot analysis because sequences at telomeres are found elsewhere in the genome (discussed above). P-element inserts into telomeric regions generate unique sequence tags and a means of assessing copy number. This has been done for P-element inserts at the

telomere of the minichromosome *Dp1187* (Karpen and Spradling 1992). We have determined the copy number of five independent insertions near the telomere of the second, third and fourth chromosomes. Our data agree with the results of the minichromosome study, showing up to a two fold underrepresentation compared with the euchromatic gene. Inserts along the length of the fourth chromosome including the insert near the telomere (line 118E-15, Fig. 3), also showed approximately twofold underrepresentation.

Concluding remarks

The mechanism underlying PEV has been a fascinating question for decades. Muller stated in 1938. "These so-called 'eversporting displacements,' do not seem, as various geneticists have thought, usually to involve actual losses, in mosaic patches, of chromosome sections containg the genes concerned." instead, he attributed the somatic variegation to "differences in the mode of formation, or rather in conformation, of the chromocentral region involved ...". Mechanistic studies have been hampered by the fact that PEV is defined by the collective behavior of individual cells, while most biochemical or molecular studies can derive information only on a population of cells. In this study we have used the *hsp26* gene, which is inducible in virtually all cell types in its normal euchromatic location. We find that the expression of *hsp26*, recovered at a variety of heterochromatic sites, mimics closely the expression seen for the *white* reporter gene present on the same P-element insert (Fig. 1). In particular, inducible expression of *hsp26* at the pericentric and fourth chromosome sites is reduced in approximately the same proportion as the loss of *white* expression. Derepression of both the *hsp26* transgene and *white* expression occurs in the presence of *Su(var)2-5^{02}* (Wallrath and Elgin 1995). We show that this loss of *hsp26-pt-T* expression (to 4% of the levels of the euchromatic gene) is not accompanied by a similar loss of DNA in diploid tissue.

The P-element inserts can also be used to monitor DNA levels following polytenization; here a consistent reduction in copy number for this set of P-element inserts in the pericentric heterochromatin is seen. Results of others suggest a complex pattern of replication during polytenization (see above). We conclude that while copy number can vary in polytene cells of *Drosophila*, this does not appear to be a necessary part o the mechanism of PEV. Other studies have indicated changes in chromatin structure (Wallrath and Elgin 1995) and changes in chromosomal organization (Wakimoto and Hearn 1990) that may be critical in establishing gene repression seen with PEV. Further investigations are needed to establish which parameters are essential for this mechanism of gene inactivation.

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