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Characterization of sequences associated with position-effect variegation at pericentric sites in *Drosophila* heterochromatin

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Abstract. In a variety of organisms, euchromatic genes brought into juxtaposition with pericentric heterochromatin show position-effect variegation (PEV), a silencing of gene expression in a subset of the cells in which the gene is normally expressed. Previously, a P-element mobilization screen identified transgenic *Drosophila* stocks showing PEV of an *hsp70-white*$^+$ reporter gene; transgenes in many of these stocks map to the chromocenter of polytene chromosome. A screen at an elevated temperature identified two stocks that under standard culture temperatures show complete repression of the *hsp70-white*$^+$ transgene. The transgenes in both cases map to the chromocenter of polytene chromosomes. Different types of middle repetitive elements are adjacent to seven pericentric transgenes; unique sequences are adjacent to two of the perimetric transgenes. All of the transgenes show suppression of PEV in response to a mutation in the gene encoding heterochromatin protein 1 (HP1). This suppression correlates with a more accessible chromatin structure. The results indicate that a pericentric transgene showing PEV can be associated with different types of DNA sequences, while maintaining a common association with the chromosomal protein HP1.

Introduction

Centric and telomeric regions of the genome are not conducive for the expression of most euchromatic genes. These regions are packaged into heterochromatin, originally defined as those portions of the genome that remain densely stained throughout the cell cycle. Heterochromatic regions generally replicate late in S-phase, are relatively gene poor, and contain a high percentage of repetitious sequences (reviewed by Weiler and Wakimoto 1995). Genes normally present in euchromatic domains placed near or within heterochromatic domains exhibit position-effect variegation (PEV), a silencing of gene expression in a subset of the cells in which the gene is normally expressed, leading to a mosaic phenotype. Examples include genes placed near pericentric or telomeric regions of *Drosophila* chromosomes (reviewed by Weiler and Wakimoto 1995; Wallrath and Elgin 1995), pericentric or telomeric regions of yeast chromosomes (Gottschling et al. 1990; Renauld et al. 1993; Allshire et al. 1994; Nimmo et al. 1994) and pericentric locations of mouse chromosomes (reviewed by Dobie et al. 1997; Kioussis and Festenstein 1997). Investigations are underway to explore the molecular mechanisms responsible for PEV. It has been suggested that the presence of repetitious DNA might be sufficient to trigger heterochromatin formation (Sabl and Henikoff 1996 and references therein).

In *Drosophila melanogaster* large blocks of pericentric heterochromatin consist of simple repeats, the satellite DNA sequences (Lohe et al. 1993). Transposable elements are also a prominent component of heterochromatin (Carmena and Gonzalez 1995; Pimpinelli et al. 1995; Sun et al. 1997; reviewed by Dimitri 1997). Transposons localize to regions containing simple satellite sequences and to distinct locations within mitotic heterochromatin (Camena and Gonzalez 1995). Transposons are frequently found as incomplete copies clustered within other mobile elements (Devlin et al. 1990; Valgeirsdóttir et al. 1990). Changes in the distribution of some transposons within heterochromatin have been noted between laboratory strains (Caizzi et al. 1993; Carmena and Gonzalez 1995). Taken together, the emerging picture is one in which *Drosophila* heterochromatin is a mosaic arrangement of different types of middle repetitive DNA and satellite DNA sequences. The data presented here suggest even further complexity.

We have recovered stocks that variegate for the expression of an *hsp70-white*$^+$ reporter gene from P-element mobilization screens (Wallrath and Elgin 1995; Wallrath et al. 1996; and this report). Expression of the *white*$^+$ gene is required for red eye pigmentation. These stocks show variation in the pattern and the severity of silencing of the *hsp70-white*$^+$ transgene. All of the variegating

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transgenes map to known heterochromatic regions: 12 localize to pericentric regions, 17 to telomeric regions, and 24 to sites along the small, mostly heterochromatic fourth chromosomes. The P-element also contains a tagged *hsp26* gene for analysis of chromatin structure. Heat shock-induced expression of the heterochromatic *hsp26* transgenes is impaired at pericentric, fourth chromosome and some telomeric sites (Wallrath and Elgin 1995; unpublished data). Using the inverse polymerase chain reaction (PCR), the DNA sequences adjacent to nine pericentric P-element inserts have been characterized. Both unique and repetitive DNA sequences were found adjacent to the variegating transgenes, suggesting that PEV does not require that the transgene be surrounded by repetitive sequences. All nine transgenes are sensitive to mutations in HP1, a prominent heterochromatin-associated protein (James et al. 1989; Eissenberg et al. 1990). A "closed" chromatin configuration observed for a pericentric transgene becomes more accessible in the presence of a mutation in HP1. This suggests that PEV reflects specialized packaging due to heterochromatin proteins that are associated with different types of sequences.

Materials and methods

Genetic manipulations. All stocks of *D. melanogaster* were cultured on standard cornmeal-sucrose medium at 25° C unless otherwise stated (Shaffer et al. 1994). To recover flies with completely repressed transgenes, females of stock 118E-X (Wallrath and Elgin 1995) carrying a P-element (including the *hsp70-white*$^+$ and *hsp26-pt-T* transgenes) on the *X* chromosome were crossed to *y* w^{67c23}*/Y*; *Sb* Δ2–3 males (containing a genomic source of transposase; Robertson et al. 1988) and cultured at 25° C. Red-eyed *Sb* male progeny were independently crossed to two *y* w^{67c23} females and the culture vial was subjected to one of two treatments: (1) a heat shock at 37° C for 1 h daily or (2) constant culture at 28° C. The two treatments gave similar increases in red eye pigmentation for stocks 39C-4 and 118E-12, two lines with *hsp70-white*$^+$ located in pericentric heterochromatin (Wallrath and Elgin 1995). Resulting non-*Sb* male progeny were screened for PEV.

To test the effects of HP1 dosage, males of the PEV stocks were crossed to females of stock *y* w^{67c23}*; Su(var)2–*5^{02}*/Cy* and cultured at 25° C. *Su(var)2–*5^{02} is a point mutation in the gene encoding HP1 (Eissenberg et al. 1992). *Cy* and non-*Cy* progeny of the same age were compared and photographed. We and others have noted that balancer chromosomes, such as the *Cy*-containing chromosome, often contain background modifiers of PEV. For those examples shown here, the *Cy*/+ progeny were phenotypically identical to hemizygous individuals in the *y* w^{67c23} background; we conclude that the *Cy* chromosome does not contain strong modifiers for these particular PEV transgenes. For chromatin structure analysis, stock 118E-10, having a transgene in pericentric heterochromatin, was crossed into stock *y* w^{67c23}*; Su(var)2–*5^{02}*/Cy* and made homozygous.

In situ hybridization. Salivary glands were dissected from late third instar larvae raised at 18° C according to Ashburner (1989). The glands were squashed using a mechanical squashing device (General Valve, Fairfield, N.I.). The P-element construct described above (Wallrath and Elgin 1995) was labeled with biotin-16-dUTP (Boehringer Mannheim) by nick translation according to Sambrook et al. (1989). The site of hybridization on the polytene chromosome was detected using streptavidin-horseradish peroxidase complex (Vector Labs) and 3,3'-diaminobenzidine (Sigma) (Lim 1993).

Eye pigment assay. Eye pigments were extracted from adults according to Khesin and Leibovitch (1978). Ten adult flies, approximately 2 to 4 days old, homozygous for a particular P-element, were homogenized in 1 ml of 0.01 N HCl in ethanol. The homogenate was placed at 4° C overnight and then warmed at 50° C for 5 min. The homogenate was centrifuged in a microcentrifuge for 10 min. The supernatant was collected and the optical density at 480 nm was recorded. To correct for background absorbance, the average value from *y* w^{67c23} was subtracted prior to reporting. Three independent samples were assayed for each stock. The average of the three values and the standard deviations are reported in Fig. 4. The values were also normalized to that of 39C-X, set at 100%, and listed as relative percent pigment in Fig. 4.

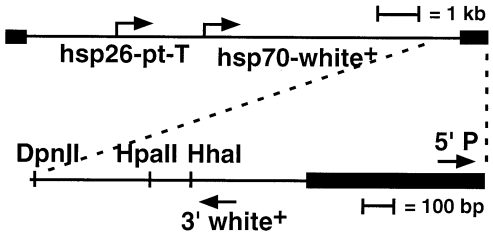

Fig. 1. Diagram of the P-element and primers used for inverse polymerase chain reaction (PCR). Shown on the *top* is a diagram of the P-element transposon used, with *filled boxes* representing the P-element inverted repeats. Enlarged at the *bottom* is a diagram showing the positions of the primers corresponding to sequences from the 3' region of the *white*$^+$ gene and from the 5' inverted repeat of the P-element (see Materials and methods). Restriction sites used to create fragments for ligation and subsequent inverse PCR are shown

Inverse PCR. Genomic DNA was isolated from each of the pericentric insertion stocks according to methods previously described (Lu et al. 1993a). Three micrograms of DNA were digested in a 30 μl volume with 60 U of enzyme. Initially the DNA was digested with *Hha*I, which cleaves within the 3' region of the *white*$^+$ gene (Fig. 1). Five microliters of the digestion reaction were used in a 150 μl volume ligation reaction with 2 U of ligase at 16° C overnight. Then 5 μl of the ligation reaction containing the circularized products were used in a PCR with primers specific for the 5' P-element inverted repeats (5' GCTTCGGCTATCGACGGGACCACCTTATGTTA 3') and specific for the 3' *white*$^+$ sequences (5'GACGAAATGAACCACTCGGAACCATTTGAGCG 3'). Buffers from the PCR Optimization Buffer kit (Invitrogen) and Amplitaq (Perkin Elmer) were used for the PCRs. Reaction products were evaluated by gel electrophoresis and used directly for cloning; 2 μl of fresh PCR product were ligated to the pCR2.1 TA cloning vector according to the manufacturer's specifications (Invitrogen). In some cases, the PCR did not yield a product, possibly indicating that the *Hha*I site in the uncharacterized flanking DNA was too far away to yield a fragment of a reasonable size for PCR. In these cases, another aliquot of genomic DNA was digested with *Hpa*II or *Dpn*II (Fig. 1) and treated as described above. Positive clones were sequenced manually using the Sanger dideoxy sequencing method with Sequenase (USB), and were also sequenced using an automated sequencing system (ABI, University of Iowa Sequencing Core Facility). At least two clones from each of the stocks were sequenced and found to have identical inserts. The occurrence of deletions and/or rearrangements during the cloning process appears unlikely, since the size and restriction maps of the cloned inserts matched those of the PCR products. The sequences of clones obtained from stocks HS-3, HS-5 and 39C-4, containing previously uncharacterized sequences, have been deposited in Genbank (Accession numbers AF068629, AF068630 and AF068631, respectively). Sequence analysis was performed using DNA Strider (Marck 1988).

Chromatin structure analysis. Nuclei were isolated from nonheat-shocked third instar larvae and treated with *Xba*I as previously described (Lu et al. 1993a). The DNA was purified, cleaved to com-

pletion with *Sal*I, separated by size on a 1% agarose-TAE gel (Sambrook et al. 1989), transferred to nylon membrane and hybridized with α-^{32}P-labeled DNA fragments (Feinberg and Vogelstein 1984) corresponding to the barley sequence (Wallrath and Elgin 1995). The amount of radioactivity on the membrane was measured using an Instant Imager (Packard Instruments). The percent cleavage at the proximal *Xba*I site was calculated as a ratio of the counts in the band generated by cleavage at the proximal *Xba*I site to the total counts in all the bands within the given lane. The values for the euchromatic transgene of stock 39C-X was set at 100%; other samples were normalized to that of 39C-X.

Results

A screen to recover completely repressed transgenes

We previously performed a P-element mobilization screen designed to recover stocks with P-element inserts in heterochromatin by scoring for PEV of an *hsp70-white*$^+$ transgene (Wallrath and Elgin 1995). The screen was carried out at 25° C, allowing only basal expression from the *hsp70* promoter; this expression is sufficient to a result in a wild-type, uniformly dark-red eye pigmentation with a single copy of this P-element in a euchromatic environment. In an initial screen of approximately 7,000 males, 3,000 transposition events were observed, 35 of which resulted in a PEV phenotype. However, heterochromatic inserts showing complete repression of the *white*$^+$ transgene have the same phenotype as flies without a P-element insert, and would have been discarded in this screen.

In order to recover stocks in which the *hsp70-white*$^+$ gene is completely silent under standard culture conditions, we repeated the P-element mobilization under conditions inducing higher levels of expression from the *hsp70* promoter, either by subjecting flies to a 1 h daily heat stock at 37° C throughout development, or by continual culture at 28° C (see Materials and methods). From 655 mobilization events, six flies showing PEV of the *hsp70-white*$^+$ transgene were recovered. These stocks were designated HS-2 through HS-7. (HS-1 showed uniform repression of the *hsp70-white*$^+$ transgene and was not included in this study.) Two of these stocks, HS-2 and HS-5, show the desired phenotype. When raised at 25° C, these stocks have essentially white eyes; when cultured with a daily 1 h heat shock at 37° C they show a small amount of pigmentation in the eye (Fig. 2). In situ hybridization to third instar larval polytene chromosomes demonstrated that the P-element inserts in stocks HS-2 and HS-5 are in the pericentric heterochromatic regions of chromosome arms *3L* and *2L*, respectively (Fig. 3). In the case of HS-2, we frequently observe two dots within the chromocenter, which may reflect a lack of chromatid pairing (Fig. 3). Similar unusual patterns of in situ hybridization to polytene chromosomes have been observed for stocks containing pericentric inserts of a *rosy*$^+$-marked P-element (Zhang and Spradling 1995).

Four of the stocks showed some red eye pigmentation when raised at 25° C. These stocks exhibit a weak PEV phenotype with increased eye pigmentation when cultured at the higher temperature. In situ hybridization to the polytene chromosomes of these stocks showed that the *hsp70-white*$^+$ transgenes of stocks HS-4, HS-6 and HS-7 are located in the telomeric region of the fourth chromosome (data not shown). The transgene in stock HS-3 mapped to the proximal banded region of *2L*, cytological region 38B (Fig. 3). The *hsp70-white*$^+$ transgenes in the three pericentric insert stocks recovered from this screen and in six pericentric insert stocks recovered from previous screens (Wallrath and Elgin 1995; Wallrath et al. 1996) were used as unique sequence entry points to clone the neighboring DNA (see below).

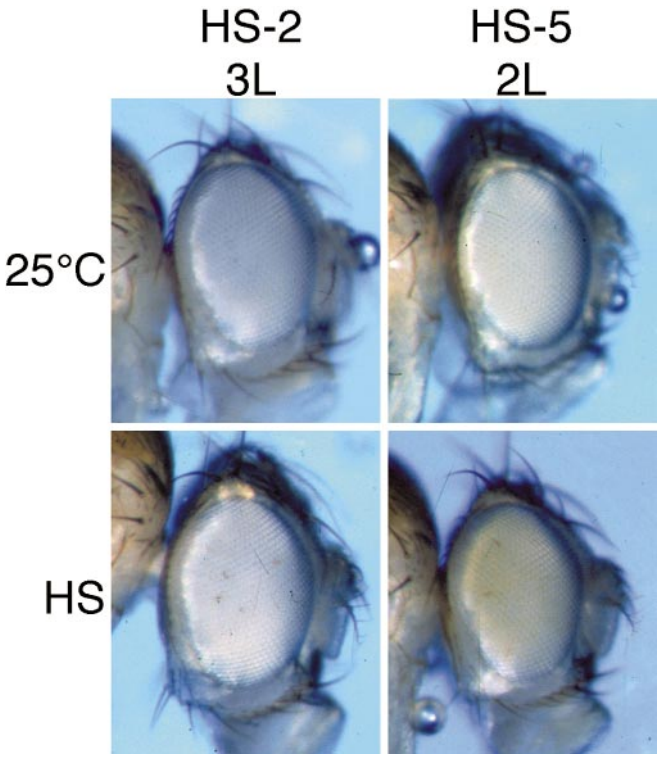

Fig. 2. Phenotypes of HS-2 and HS-5, showing temperature dependence of eye coloration. Stocks HS-2 and HS-5 carry single inserts of the P-element in the pericentric regions of *3L* and *2L*, respectively. Stocks were cultured at 25° C or given a daily 1 h 37° C heat shock (*HS*) treatment to induce expression of the *hsp70-white*$^+$ promoter

Eye pigment assays performed with pericentric insert stocks show a range of *hsp70-white*$^+$ expression (Fig. 4). Six stocks (39C-3, 118E-10, HS-2, 39C-4, 118E-12 and HS-5) showed severe repression of the *hsp70-white*$^+$ transgene. Pigment values for these stocks are less than 10% compared with the values obtained for stock 39C-X, in which the transgene is present at a euchromatic location. Stock 39C-X shows uniform, dark-red eye pigmentation (Wallrath and Elgin 1995). Stocks 118E-25, HS-3 and 39C-2 showed intermediate levels of pigment ranging from 22% to 86% compared with the 39C-X value. The quantitative values obtained correlate roughly with the percentage of pigmented ommatidia as estimated by observation.

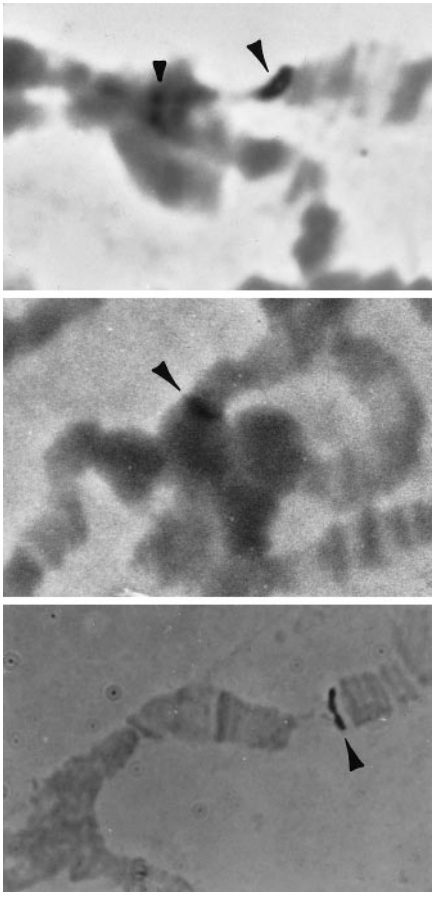

Fig. 3. In situ hybridization to polytene chromosomes. Chromosomes from stocks HS-2, HS-5 and HS-3 are shown (*top* to *bottom*). The transgenes of stocks HS-2 and HS-5 map to the chromocenter. The site of insertion is indicated by a *large arrowhead*. A *small arrowhead* denotes the two dots of hybridization frequently seen in stock HS-2 (see text for discussion). The P-element insert in stock HS-3 is located at 38B. The probe was the entire P-element plasmid in all cases (Wallrath and Elgin 1995)

Pericentric transgenes are immediately adjacent to repetitive and unique DNA sequences

To examine the DNA sequences associated with transgenes exhibiting PEV, inverse PCR (Ochmann et al. 1988) was employed to clone the DNA adjacent to the inverted repeat of the 5' P-element. The sequences of the adjacent DNA were determined and compared with sequences in Genbank by performing a BLAST search. A schematic diagram of the sequenced regions is shown in Fig. 4. Sequences showing greater than 85% identity to a known sequence are labeled accordingly.

Both unique and repetitive DNA sequences are found in the DNA adjacent to the pericentric inserts on chromosome *2*. At least 1.1 kb of unique DNA is adjacent to the transgene in stock HS-5. The unique nature of the DNA was confirmed by experiments showing that this fragment hybridizes to a single band on a Southern blot of genomic DNA (data not shown). This sequence is 62% A+T and does not contain any substantial open reading frames or repeats. The transgene in stock HS-3 is inserted adjacent to a short stretch of unique DNA. Immediately following the unique sequences are sequences with 85% identity to a portion of the 0.4 kb subtelomeric minisatellite DNA sequences of chromosome arm *2L* (accession no. U35404; Walter et al. 1995), and sequences with 83% identity to a portion of the 1.8 kb telomere-associated sequences (TAS) of a mini-*X* chromosome (accession no. L03284; Karpen and Spradling 1992). The entire region is 98% identical to the sequence of a subclone from the P1 clone DS0083 (accession no. AC000773, Berkeley *Drosophila* Genome Project). This P1 clone maps to region 38B, the site of P-element insertion in stock HS-3. A 146 bp stretch of DNA that is 87% identical to the 1.8 kb TAS is also present flanking the transgene of stock 39C-3. TAS and non-TAS sequences within this clone are similar to those found adjacent to *xanthine dehydrogenase*

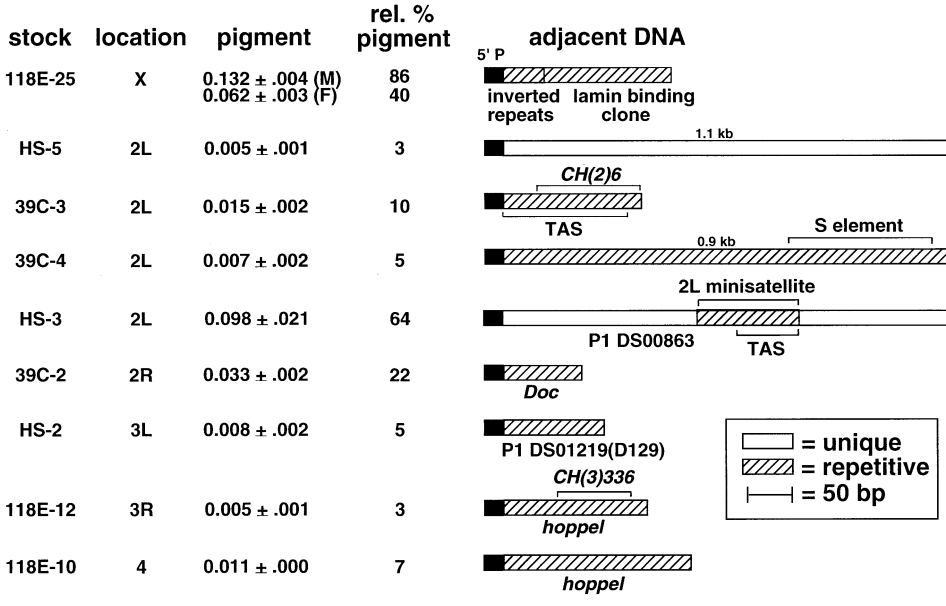

Fig. 4. Percent eye pigment and DNA sequence adjacent to the pericentric transgenes. The amount of eye pigment was determined and normalized to that of stock 39C-X, containing a euchromatic transgene (see Materials and methods). For stock 118E-25 with a pericentric *X*-chromosome transgene, values for males (*M*) and females (*F*) are reported separately. The cloned DNA flanking the 5' P-element inverted repeat is shown as an *open box* (unique sequence) or *shaded box* (repetitive DNA). The *solid box* represents the 5' P-element inverted repeat. Regions showing similarity to known DNA sequences are indicated (see text for details)

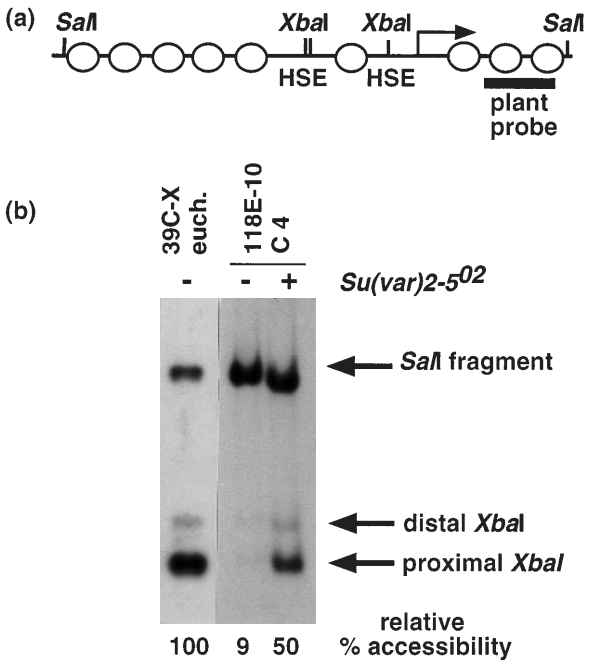

Fig. 5a, b. Effects of an HP1 mutation on chromatin structure of a pericentric *hsp26* transgene. **a** Diagram of the *hsp26* promoter region: *HSE* heat shock elements, *circles* nucleosomes. **b** Southern blot analysis showing the accessibility of the proximal *Xba*I site in a euchromatic transgene (*39C-X, euch.*) and a fourth chromosome pericentric transgene (*118E-10, C4*) with (+) and without (−) a mutation in HP1 [*Su(var)2–5*^{02}]. Relative percent cleavage of the proximal *Xba*I site is shown below each lane with the value for a euchromatic *hsp26* transgene set at 100%

(*rosy*$^+$)-marked P-element insert, *CH(2)6* (accession no. L36595; Zhang and Spradling 1995).

The transgene of stock 39C-4 is adjacent to repetitive DNA sequences containing a 239 bp region that shows 90% identity to a *Drosophila* S-element (accession no. U33470; Kidwell 1993). The S-element is a member of a diverse family of inverted repeat-containing transposons that map to the chromocenter and several sites within euchromatin (Kidwell 1993; Merriman et al. 1995). When the cloned DNA is used as a probe on a Southern blot of genomic DNA (cleaved with restriction enzymes that do not cut within the clone), four strongly hybridizing bands and several weaker smeared regions are visualized (data not shown). A similar pattern of hybridization is seen when sequences similar to the S-element are removed from the probe (data not shown). This suggests that the sequences adjacent to the 5' P-element inverted repeat include an uncharacterized repetitive element. Overall, this cloned sequence is 63% A+T and does not contain any long open reading frames or repeats. Lastly, the transgene in stock 39C-2, also located on the second chromosome, is flanked by sequences that are 98% identical to the *Doc* transposon (O'Hare et al. 1991, discussed below).

P-element inserts recovered in the pericentric heterochromatin of chromosome *3* are within repetitive DNA sequences. Adjacent to the strongly repressed transgene in stock HS-2 is 107 bp that is completely identical to the sequence within the P1 clone DS01219 (D129) (accession no. AC004244, Berkeley *Drosophila* Genome Project) that maps to region 35B4-35C1 on larval salivary gland polytene chromosomes. Therefore, this sequence is present in at least two copies within the genome. Several attempts were made using other enzymes to clone additional flanking DNA by inverse PCR to obtain a larger probe for Southern blot analysis, but all failed. Stock 118E-12 shows strong PEV; the adjacent DNA is 88% identical to the *hoppel* transposon (accession no. X78388, Kurenova et al. 1990). A fragment of this clone also shows 91% identity to a region adjacent to a *rosy*$^+$-marked P-element on the third chromosome, *CH(3)336* (accession no. L36596; Zhang and Spradling 1995). In addition, *hoppel* sequences are also found adjacent to the pericentric fourth chromosome insert in stock 118E-10 (Fig. 4).

The *hsp70-white*$^+$ transgene of stock 118E-25, located on the *X* chromosome, shows dosage compensation despite its location in heterochromatin. Males express twice the amount of eye pigment as do females (Fig. 4). This is in contrast to what has been seen for an *Hsp82* transgene. The *Drosophila pseudoobscura Hsp82* gene transformed into *D. melanogaster* shows dosage compensation were present at *X*-linked euchromatic sites, but not when present at *X*-linked heterochromatic sites (Arkhipova et al. 1997). The DNA immediately adjacent to the *hsp70-white*$^+$ transgene in stock 118E-25 shows 95% identity to the inverted repeat sequences from genomic clone λ20p7, which hybridizes to *Drosophila* heterochromatin (accession no. L10989; Baiborodin et al. 1993). Adjacent to these inverted repeats are sequences that are 92% identical to those present within clone λ20p1.4. Fragments of this clone bind to polymerized *Drosophila* nuclear lamin in vitro (Baricheva et al. 1996). In situ hybridization to salivary gland polytene chromosomes in intact cells with the λ20p1.4 clone shows localization to the nuclear periphery, the region where heterochromatin residues (Baricheva et al. 1996; reviewed in Marshall 1997).

We made several attempts to clone the DNA sequences adjacent to the 3' P-element inverted repeat using a similar strategy to that employed for the sequences adjacent to the 5' P-element inverted repeat. Multiple attempts using different restriction enzymes were unsuccessful. This might reflect a lack of restriction enzyme recognition sites within these regions, suggesting that simple repetitious sequence could be present.

PEV transgenes adjacent to repetitive and unique DNA sequences respond to mutations in HP1

Given that both repetitive and unique DNA sequences are associated with the variegating transgenes, we asked whether or not changes in the dosage of HP1 (James et al. 1989; Eissenberg et al. 1990) would have an effect on the degree of PEV in all cases. Females carrying *Su(var)2–5*^{02}, a point mutation in the gene encoding HP1 (Eissenberg et al. 1992), were crossed to males of the different

variegating stocks. In all cases, PEV was suppressed (Wallrath and Elgin 1995; data not shown).

The chromatin structure of a heterochromatic transgene is more accessible in an HP1 mutant background

The *hsp26* gene, in its endogenous euchromatic location, has two nucleosome-free regions upstream of the transcription start site (Thomas and Elgin 1988). These regions map to the location of the heat shock elements (HSEs). *Xba*I sites within the *HSE*s have been used quantitatively to measure accessibility within the promoter region in isolated nuclei (Lu et al. 1993b, 1995; Wallrath and Elgin 1995). *hsp26* transgenes at heterochromatic locations have an altered chromatin structure (Wallrath and Elgin 1995). Here we examine the accessibility of the proximal *Xba*I site in a wild-type and HP1 mutant background. Of all the pericentric stocks, 118E-10 shows the greatest contrast in eye pigmentation and *hsp26*-induced expression in the presence and absence of an HP1 mutation (Wallrath and Elgin 1995). Therefore, we chose this stock to examine the chromatin structure. In a wild-type background, the proximal *Xba*I site within the *hsp26* promoter region of this pericentric transgene is 9% accessible compared with a euchromatic *hsp26* transgene. We attribute this reduction in accessibility to the packaging of heterochromatin. In an HP1 mutant background this value increases to 50% (Fig. 5). This data directly demonstrate an opening of the chromatin structure that correlates with a mutation in HP1.

Discussion

The molecular mechanisms underlying the formation of heterochromatin remain a mystery. Nonetheless, we are beginning to identify DNA sequences and proteins that play a role in the process of heterochromatin formation (Lohe et al. 1993; Le et al. 1995; Cléard et al. 1997; reviewed by Elgin 1996; Wallrath 1998). The formation of heterochromatin has been postulated to start at "initiation" sites and spread to "termination" sites (Locke et al. 1988; reviewed by Eissenberg 1989). However, no such sequences have been identified to date, and the question of heterochromatin spreading has been widely debated (reviewed by Karpen 1994; Henikoff 1996). Our collection of stocks with pericentric *hsp-70-white*$^+$ transgenes serves to provide entry points for a molecular characterization of heterochromatin.

Heterochromatin makes up 30% of the *Drosophila* genome, with the majority estimated to be simple satellite sequences (Lohe et al. 1993). Many of the simple satellite sequences show dramatic underrepresentation in salivary gland polytene chromosomes (reviewed by Weiler and Wakimoto 1995). We have shown that the transgenes in several of the PEV stocks showing strong variegation (such as 118E-12 and 39C-4) are dramatically underrepresented in salivary gland polytene nuclei, while others, showing modest PEV (such as 39C-2 and 39C-3) are only slightly underrepresented (Wallrath et al. 1996). Pericentric transgenes are less accessible to restriction enzyme digestion in isolated nuclei (Wallrath and Elgin 1995; Fig. 5). This inaccessibility might be due to packaging of the transgene into a very regular nucleosome array, as seen for the heterochromatic transgenes of stocks 39C-4 and 118E-12 (Wallrath and Elgin 1995). Repetitive elements, including the 1.688 gm/cm^3 satellite sequences from *Drosophila*, are similar in sequence and DNA bending characteristics to sequences shown to position nucleosomes in vitro (Fitzgerald et al. 1994). A library made from isolated mouse mononucleosomal DNA shows enrichment of simple di-, tri- and tetra-repeats that hybridize to centromeres (Widlund et al. 1997). Therefore, we reasoned that the pericentric transgenes might be surrounded by simple satellite DNA sequences. However, this was not the case; the transgenes are adjacent to unique and middle repetitive DNA sequences, not satellite DNAs.

In situ hybridization to metaphase chromosomes shows that transposable elements are a significant component of heterochromatin (Carmena and Gonzalez 1995; Pimpinelli et al. 1995; Sun et al. 1997). We have identified two transposable elements associated with variegating transgenes, *Doc* and *hoppel. Doc* is a LINE-like transposable element closely related to *I-, F-,* and *G*-elements and *jockey* (O'Hare et al. 1991). *Doc* is found in clusters near the primary constriction and throughout the heterochromatin of all *D. melanogaster* chromosomes (Carmena and Gonzalez 1995; Pimpinelli et al. 1995); in particular, it is found in the 412 kb that make up the fully functional centromere of the mini-*X* chromosome *Dp1187* (Sun et al. 1997). There are an estimated 30 copies of *Doc* within euchromatin (Pimpinelli et al. 1995). *hoppel*, also called *1360*, has been placed in the same class of transposable elements as the P-element and *Hobo* (Kurenova et al. 1990). *hoppel* sequences are estimated to be present at 10 to 30 sites in euchromatin and throughout heterochromatin. *hoppel* probes hybridize strongly to four or five bands along the fourth chromosome, including the telomeric region. In addition, hybridization is seen throughout the chromocenter and the proximal region of the euchromatic arms (Kurenova et al. 1990).

We recovered two pericentric inserts located adjacent to copies of the TAS. Telomeric sequences are also found in centric regions of mice and plant chromosomes; it has been speculated that these might have arisen by specific sequence interchanges between the two regions (Yen et al. 1995; Presting et al. 1996). Subtelomeric TAS elements of *Drosophila* have been postulated to be hot spots for P-element insertion on the mini-*X* chromosome *Dp1187* (Karpen and Spradling 1992). Interestingly, TAS sequences are found flanking several variegating *hsp70-white*$^+$ transgenes located at the telomeres of chromosomes *2R* and *3R* (L. Wallrath, M. Pavlova, R. Levis, H. Biessmann, S. Elgin, unpublished data). These transgenes do not show suppression of PEV in the presence of a mutation in HP1 (Wallrath and Elgin 1995). Thus, TAS elements are associated with gene silencing at both pericentric and telomeric locations, but only transgenes at pericentric locations respond to HP1 mutations. This suggests that the DNA immediately flanking the transgene is not the sole element determining the mode of silencing.

Sequences more distal to the insertion site and/or the chromatin structure of a given domain might dictate the type of silencing observed.

To date, there have been a few reports in which the DNA sequences adjacent to a variegating gene have been characterized. Stock *In(1LR)pn2a* contains an inversion in which the euchromatic breakpoint disrupts the *vinculin* gene, placing it adjacent to AAGAG satellite DNA sequences (Tolchkov et al. 1997). Neighboring genes *pn, wapl, Pgd* and several vital loci variegate owing to the juxtaposition of a large block of centric *X*-chromosome heterochromatin. A secondary rearrangement that displaces a portion of the heterochromatin causes a suppression of PEV, despite the retention of approximately *1* Mb of satellites DNA sequences. In this case, satellite DNA sequences alone are insufficient to induce PEV. In contrast, variegation of the *brown* allele bw^D is apparently due to an insertion of approximately 1.5 Mb of the satellite DNA sequence AAGAG within the coding region of the gene (Platero et al. 1998). This allele causes *trans*-inactivation of a paired $brown^+$ homolog; this *trans*-inactivation is also sensitive to the distance from a large block of heterochromatin (reviewed by Henikoff 1997).

In addition to satellite DNA, transposons have been found at heterochromatic breakpoints that induce PEV. Three chromosomal rearrangements that cause variegation of the $white^+$ gene have generated new junctions with sequences similar to a Type I mobile element (Tartoff et al. 1984). Transposons and novel A-T-rich sequences are adjacent to $rosy^+$ P-element inserts (Zhang and Spradling 1995). Two of the sequences reported from that study, *CH(2)6* and *CH(3)336*, match those found adjacent to the *hsp70-*$white^+$ inserts reported here (Fig. 4). Unlike the PEV inserts described here, all of the pericentric autosomal $rosy^+$ inserts examined were found to be fully represented in polytene chromosomal DNA (Zhang and Spradling 1995). Thus, the same heterochromatic sequences can be replicated to different extents depending on their position within heterochromatin.

Why have we and others not recovered transgenes adjacent to satellite DNA sequences? Perhaps these sequences are "cold spots" for P-element insertion due to the nature of the DNA sequence. It is also possible that the satellite DNA sequences are packaged into a chromatin structure that renders the DNA inaccessible to P-element integration. It is worth noting that examples in which satellite sequences are adjacent to variegating genes are the result of chromosomal rearrangements. DNA breaks due to irradiation might be more likely to occur in satellite DNA than are the DNA insertion events associated with P-element transposition.

The findings reported here, and those of others cited above, indicate that a wide range of DNA sequences, unique, middle repetitious and satellite, can be associated with variegating gene expression. An examination of gene silencing in a variety of organisms suggests that chromosomal proteins that influence heterochromatin might be more highly conserved than the underlying DNA. HP1, first characterized in *Drosophila* (James et al. 1989) has been identified as a heterochromatic protein in mice and humans (Singh et al. 1991; Saunders et al. 1993; Wreggett et al. 1994). Homologs of other suppressors and enhancers of PEV [including En(z), Rpd3 and SU(VAR)3–9] have been identified in yeast and mammals (De Robertis et al. 1996; Laible et al. 1997; Laible and Jenuwein, personal communication). Perhaps most remarkable is the ability of the mammalian homolog of *Enhancer of zeste* to participate in gene silencing both in *Drosophila* heterochromatin and at *Saccharomyces cerevisiae* telomeres (Laible et al. 1997).

In this study all of the variegating transgenes characterized, regardless of their site in the pericentric region or of the nature of the adjacent DNA, show a suppression of PEV in response to a mutation in the gene encoding HP1. In at least one case, this suppression is associated with an opening of the chromatin structure. Prior to this observation, an effect on chromatin structure was inferred on the basis of changes in gene expression. These data support the conclusion that the general and defining characteristics of the heterochromatic state are based on the associated proteins, presumably generating an alternative chromatin structure, rather than on a specific set of DNA sequences. Further studies of the chromatin structure of genes silenced by heterochromatin are underway.

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