An investigation of heterochromatin domains on the fourth chromosome of

*Drosophila melanogaster*

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ABSTRACT (200 words)

The banded portion of *Drosophila melanogaster* chromosome four exhibits euchromatic and heterochromatic characteristics. Reminiscent of heterochromatin, it contains a high percentage of repetitive elements, does not undergo recombination, and exhibits high levels of HP1 and histone 3 lysine 9 dimethylation. However, in the distal 1.2Mb, the gene density is typical of euchromatin, and this region is polytene in salivary gland nuclei. Using *P* element reporters carrying a copy of *hsp70-white*, alternative chromatin packaging domains can be distinguished by the eye color phenotype. Mapping studies identified the repetitive element 1360 as a candidate for heterochromatin targeting in the fourth chromosome *Hcf* region. We report here two new screens using this reporter to look for additional heterochromatin target sites. We confirm that reporter elements within 10kb of 1360 are usually packaged as heterochromatin; however, heterochromatin packaging occurs in the *sv* region in the absence of 1360. Analyses of the sequences adjacent to *P* element reporters show no simple association between specific repeated elements and transgene expression phenotype on a whole chromosome level. The data require that heterochromatin formation as a whole depends on a more complex pattern of sequence organization rather than the presence of a single sequence element.
INTRODUCTION

DNA is present in the cell nucleus in two main conformations: euchromatin and heterochromatin. These two states differ in a number of characteristics, many of which relate to the assembly of the nucleoprotein complex consisting of DNA, histones and other chromatin-associated proteins (for a review see GREWAL and ELGIN 2002). Classically, euchromatin and heterochromatin are distinguished based on staining patterns during the cell cycle; euchromatin undergoes cycles of condensation and decondensation, while heterochromatin remains densely staining throughout (HEITZ 1928). This observation is thought to reflect the fact that heterochromatin is packaged in a highly regular nucleosome array which presumably forms stable higher order structures (SUN et al. 2001). Heterochromatin is also associated with biochemical marks that differ from those of euchromatin. Heterochromatin is rich in heterochromatin protein 1 (HP1) (JAMES et al. 1989), and histone 3 di- or tri-methylated at lysine 9 (H3K9me2/3), while euchromatin shows high levels of acetylated histones H3 and H4, as well as spikes of histone 3 tri-methylated at lysine 4 (H3K4me3) at the promoters of active genes (reviewed in MARTIN and ZHANG 2005).

The biochemical differences between heterochromatin and euchromatin are reflected also in other characteristics. Heterochromatin exhibits severely reduced levels of recombination and replicates late during S-phase in comparison with the bulk of the euchromatic sequences. While euchromatin contains the majority of the gene space, heterochromatin generally contains a high density of repeated elements such as remnants
of retroviruses and DNA transposons (for a review see DIMITRI et al. 2005). In contrast to the abundance of transcriptionally active sites in euchromatin, heterochromatin is mainly transcriptionally silent; however, those few genes naturally embedded within heterochromatic domains (ca. 2% of the total; SMITH et al. 2007) are developmentally regulated and actively transcribed irrespective of this position.

In Drosophila melanogaster, the phenomenon of position effect variegation (PEV) can be used as a convenient assay to determine the chromatin state at a particular position in the genome. PEV describes the observation that if a euchromatic gene is brought into close proximity with heterochromatin, the heterochromatic structure can spread across the gene, thus silencing it (LOCKE et al. 1988; MULLER 1930). This spreading is to some degree stochastic, and thus a variegating phenotype is observed, as some cells carry a silent copy of the gene while others carry an active copy. Monitoring for PEV has been used in the past to probe the chromatin environment of the fourth chromosome of D. melanogaster. The fourth chromosome is a small acrocentric chromosome with an entirely heterochromatic right arm and a 1.2 Mb left arm that exhibits characteristics of both heterochromatin and euchromatin. This domain is highly enriched for HP1 (JAMES et al. 1989) and H3K9me2 (HAYNES et al. 2004; SCHOTTA et al. 2002), but nonetheless shows a gene density similar to the other euchromatic chromosome arms in D. melanogaster (SLAWSON et al. 2006). The fourth chromosome has a much higher repeat density than the other euchromatic chromosome arms and is repressed in recombination (BARTOLOME et al. 2002; KAMINKER et al. 2002; LOCKE et al. 1999; MIKLOS et al. 1988; SLAWSON et al. 2006).
Using a PEV assay that employs a \( P \) element-derived reporter containing the \textit{white} gene (which confers red eye color) driven by an \textit{hsp70} promoter, the chromatin domain structure of the \textit{D. melanogaster} fourth chromosome has been systematically characterized. This research demonstrated that the distal banded portion of the fourth chromosome is largely heterochromatic (as most reporters show a variegating phenotype), but includes at least three euchromatic domains (where reporters show a full red eye phenotype) (\textit{Sun et al.} 2004). Analysis of the region surrounding the \textit{Hcf} gene at 102B has implicated a specific transposable element remnant, 1360, in the nucleation of heterochromatin formation (\textit{Sun et al.} 2004). In a direct test, a copy of 1360 has been shown to promote silencing of an adjacent promoter (\textit{Haynes et al.} 2006). However, several regions of the fourth chromosome have few or no copies of 1360, suggesting that other elements or conditions play a role. In the studies presented here, we expand the characterization of the fourth chromosome chromatin domains by two specific screens, one examining the region around \textit{Hcf} (which is largely heterochromatic), and one examining the region around \textit{sv}, which is packaged as euchromatin. We confirm the domain map generated by previous studies for the \textit{Hcf} region of chromosome four, documenting a small euchromatic domain upstream of the \textit{lgs} gene, and refine the limits of the domain adjacent to \textit{sv}. The results confirm a pattern of interspersed heterochromatic and euchromatic domains on the fourth chromosome. While the additional data support a role for 1360 in targeting heterochromatin formation in the region around \textit{Hcf}, other repetitious sequences must serve this role in the heterochromatic
region proximal to the \textit{sv} gene at 102D, supporting a more complex model for the chromosome as a whole.
MATERIALS AND METHODS

All flies used in the experiments described herein were grown on standard sucrose-corn meal media supplemented with yeast granules (SHAFFER et al. 1994). Growth conditions were 25°C and 70% humidity.

*P element screen focusing on the genomic region adjacent to Hcf*

The *P* element reporter construct is illustrated in Figure 1A, while the crossing scheme employed is shown in Figure 1B. In essence, this genetic screen is identical to screen 2 described by Sun and colleagues (SUN et al. 2004). Briefly, the 39C-12 *P* element (located on the fourth chromosome just upstream of *Hcf*) was mobilized by crossing virgin females homozygous for the *P* element to males carrying an active transposase on chromosome 3, marked with the visual dominant marker *Sb* (*SbΔ2-3*). Male progeny carrying both the transposase and the *P* element were selected and crossed to virgin females of MMR (multiple marked recessives), a line carrying visual recessive alleles on each chromosome (cross 2 in Figure 1B). The transposase present in the males of cross 2 has the potential to cause the *P* element to transpose to a new location in the germline of these males. Male progeny of cross 2 with the *P* element and the desired eye phenotype but without the transposase were selected; they were also required to be homozygous for *spadpol*, as this phenotype would indicate that the *P* element no longer resides on the 39C-12 fourth chromosome. This strategy made it possible to retain males from cross 2 with both red and variegating eye color as these represent either true transposition events or *P* element mediated recombination between the two homologs. The selected males were crossed to MMR virgin females (cross 3) to map the location of their *P* elements to one
specific chromosome. Only individuals with a new $P$ element insertion on chromosome four or chromosomal rearrangements of the fourth chromosome were retained for further molecular analysis.

**$P$ element screen focusing on the genomic region adjacent to $sv$**

The crossing scheme is shown in Figure 1C. Mobilization of the $P$ element (see construct map Figure 1A) from the starting line 4-M1030 (which carries the $P$ element inserted in the $sv$ gene) was carried out as described for the first screen above. Virgin females heterozygous for the $P$ element insertion (the second copy of the fourth chromosome was discernable by a dominant marker, $c^{D}$) were crossed to males of a line carrying the transposase marked with $Sb$ ($SbΔ2$-3). From the progeny, males carrying both the transposase and the $P$ element were collected to be used in cross 2. These males were crossed to virgin females of MMR. From the progeny of cross 2, males were selected that no longer carried the transposase, but that retained the $P$ element and showed the desired eye phenotype (a switch from red to variegating). These males were crossed to MMR virgins in cross 3 to map the location of their $P$ elements to one specific chromosome. Only individuals with a new $P$ element insertion or chromosomal rearrangements on chromosome four were retained for further analysis.

**Eye pigment analysis**

Selected $P$ element insertion lines were crossed to a $yw/yw$; $+/+$; $+/+$; $+/+$ stock as well as to a $+/+$; $CyO-GFP/Su(var)2-5^02$; $+/+$; $+/+$ stock. The cross to the $yw$ stock allowed the eye phenotype induced by the $P$ element insertion to be assayed in a common genetic
background, while the cross to the $Su(var)2-502$ stock made it possible to assess the
susceptibility of the $P$ element to a known suppressor of variegation. Male and female
flies carrying the $P$ element from the $yw$ cross were photographed with a Pixelink digital
camera mounted on a Nikon SMZ-U dissecting microscope, as were pictures of males
and females carrying the $P$ element insertion and the $Su(var)2-502$ allele from the second
cross.

Eye pigment levels were assayed in triplicate by extracting pigment from 5-10 three-day-
old flies in a solution of 10mM HCl in ethanol. Males and females were assayed
separately. The flies were homogenized in 500µl extraction buffer and incubated at 50°C
for 10 min. The debris was removed by centrifugation (10 minutes, 13,000g) and the
supernatant transferred to a new tube. Eye pigment levels were measured by
spectrophotometry at 480nm (adapted from KHESIN and LEIBOVITCH 1978).

**Mapping of $P$ element insertions**

Mapping of $P$ element insertion sites from lines recovered in both screens was carried out
in two stages. The first stage is designed to check the integrity of the $P$ element
transgene and to eliminate lines carrying multiple $P$ element insertions, while in the
second stage the genomic location of the insertion site is determined (based on (SUN *et al.*
2004).

Genomic DNA was isolated from adult flies by homogenization in grinding buffer (0.2M
sucrose; 0.1M TRIS; 0.1M NaCl; 0.05M EDTA; 0.5% SDS; pH 9.2). The homogenate
was incubated at 65°C for 30 to 60 minutes. After addition of potassium acetate (pH 10) to a final concentration of approximately 1M, the samples were incubated on ice for 30 minutes. Debris was removed by centrifugation (10 min. at 10,000g). An equal amount of ethanol was added to the supernatant and the DNA precipitated by centrifugation (10 min. at 13,000g). The DNA pellet was washed with 70% ethanol, air dried and resuspended in 1xTE. This procedure was followed by a phenol/chloroform extraction and a second ethanol precipitation.

Three sets of digests followed by Southern blots were carried out for each line to check the integrity of the P element transgene and to determine insert copy number (for details see also SUN et al. 2004). The probe used in all blots corresponds to the plant fragment downstream of the hsp26 promoter, unique in the D. melanogaster genome (see Figure 1A). A SalI digest was used to assay a fragment internal to the P element transgene, checking for the integrity of the hsp26-plant region (see Figure 1A). A digest with BglII was used to assay the integrity of the plant fragment and its upstream sequences, as BglII cuts between the plant fragment and the hsp70 promoter (see Figure 1A). This BglII digest also determines whether the 3’P end of the transgene is still in its original location. This assay is important in determining whether the P element excised completely or if only one end has moved, generating a genomic deletion or duplication. A second digest with XbaI, which cuts once within the transgene at a site upstream of the plant fragment, assayed the integrity of the plant fragment and the sequences downstream of it; this assay also determines whether the 5’P end is still in its original location (see Figure 1A).
Digests of 10µg of genomic DNA from each P element insertion line were carried out according to the manufacturer's recommendations for BglII, XbaI and SalI (New England Biolabs). The digested DNA was size fractionated by agarose gel electrophoresis (1% agarose, 1x TAE). Southern blots were prepared using an alkaline transfer method, and hybridization was carried out using phosphate-SDS buffer (SAMBROOK and RUSSELL 2001). Radiolabeled probes corresponding to the plant fragment were generated by random priming using the Megaprime DNA labeling system (Amersham/GE Healthcare). The size of fragments detected in the new P element insertion lines was compared to that of the parent lines. Multiple fragments detected per lane indicated that there were multiple P elements present in the line, while the detection of no fragment, or of fragments considerably smaller than the parental fragments (particularly for the internal SalI fragment), indicated that the P element was damaged or lost. Lines exhibiting the latter patterns were excluded from further analysis.

Inverse PCR was used to map the exact genomic locations of P elements that had transposed or caused rearrangements as described in (SUN et al. 2004). Due to technical limitations, only DNA proximal to the 5' end of the P element could be mapped with this technique. Briefly, genomic DNA was digested with three enzymes, HhaI, HpaII and DpnII (New England Biolabs), each having a cleavage site close to the 5' end of the P element. Enzymes and salts were removed from the samples with the QIAquick PCR purification kit according to the manufacturer's recommendations (QIAGEN). The digested DNA was circularized with T4 DNA ligase (New England Biolabs). The circularized DNA served as template for inverse PCR to map the 5' end of the P element.
(primer sequences: 5'-AGA CGA AAT GAA CCA CTC GGA ACC-3’ and 5’- CTT CGG CTA TCG ACG GGA CCA CCT TA-3’). PCR products were sequenced using BigDye terminator technology (ABI), and the exact insertion sites were determined by BLAST (ALTSCHUL et al. 1990) searches against the D. melanogaster genome sequence (release 5.1). Both 3’ and 5’ ends of the P elements were confirmed by PCR with primers specific to the new genomic location.

**Statistical analysis**

Statistical analyses were carried out using PERL scripts as well as SAS 9.1.3 (SAS Institute Inc.). Based on the natural transposon annotation from FlyBase (release 5.1 of the D. melanogaster genome), the fraction of nucleotides occupied by a particular repeated element in the sequence surrounding a specific P element insertion was calculated for all independent insertions shown on the map in Figure 2C using custom PERL scripts. The window size surrounding the P element insertion was increased in 1kb increments from 1kb to 50kb. In addition, the full data set described above was trimmed to contain only data from transgene insertions more than 10kb apart from each other. Both the original data set and this trimmed data set were used to conduct a permutation analysis to determine if the mean number of nucleotides in any given window occupied by a certain natural transposon differed between red and variegating lines (SAS using PROC MULTTEST, mean test). This analysis was carried out for each repeated element present on chromosome four based on the annotation provided by FlyBase (release 5.1). Significance levels were adjusted for multiple testing by the Bonferroni method.
To investigate the spatial relationship between the repetitive element 1360 and the P element induced eye color phenotype, the distance to the closest 1360 element 60bp or larger, annotated by FlyBase (release 5.1) was calculated for each P element insertion site. The 60bp cut-off was chosen to a) make this new analysis comparable to the one reported by Sun and colleagues (Sun et al. 2004), and b) because the chance of false positive matches is highly increased for small fragments.
RESULTS

Chromatin domains on the fourth chromosome near Hcf

We used a P element mobilization screen to further investigate the region on chromosome four of Drosophila melanogaster adjacent to Hcf, a region where previous data have indicated that euchromatic and heterochromatic domains are in close proximity (see the insertion map in Figure 2C) (Sun et al. 2004). Line 39C-12, carrying a reporter just upstream of Hcf (nucleotide position 380,116), was selected as the starting point for this screen. Its P element construct, which carries a copy of white under the control of the hsp70 promoter (see Figure 1A for a map of the construct), conveys a variegated eye color pattern due to PEV causing partial silencing of the transgene. The goal of the screen was to recover transposition events leading to a change in eye phenotype from variegated to red in order to further demarcate the chromatin domains in this region.

Based on the crossing scheme shown in Figure 1B, 2,873 single male crosses were screened, resulting in the recovery of 1,449 males which harbored putative mobilization events based on their overall phenotype (transposition rate ~50%). These males exhibited the following eye color phenotypes: 1,378 males with solid red eye color (95%), 42 with non-red solid eye color (3%), and 28 males with variegating eye color (1%). Where transposition occurred, 44% (586) and 54% (719) of the cases mapped to chromosomes two and three respectively, and 7 lines (0.5%) had insertions that mapped to the Y chromosome. 18 lines (1.4% of the total) carried reporters that mapped to chromosome four and were the subject of further study. These frequencies are similar to those reported earlier for screens of this type (Sun et al. 2004).
Mapping results for the 18 fourth chromosome lines with \( P \) element reporter insertions from Southern blot analysis and inverse PCR are summarized in Table 2 and in Figure 2A. Two lines exhibiting red eye color were excluded from further analysis, as the \( P \) element had been damaged during the transposition event. Seven true transposition events (labeled "jumps" in Table 2) were recovered. Transposition 7-M201 (exhibiting a full red eye) confirms the presence of a new euchromatic domain upstream of \( lgs \), previously only inferred from deletion lines. Additional lines were generated by partial mobilization events of the 5' end of the \( P \) element that resulted in small genomic deletions and duplications adjacent to the original \( P \) element.

In previous studies, the closest euchromatic domain distal to the site of the variegating insertion line 39C-12 (nucleotide position 380,116; cytological band 102B5) was identified by line 4-M1285 (nucleotide position 522,199; cytological region 102C1; see also Figure 2C) (Sun et al. 2004). However, \( P \) element generated genomic deletions adjacent to the insertion in 39C-12 demonstrated that the deletion of approximately 22kb is sufficient to change the eye phenotype from variegating to red (Sun et al. 2004). In this study, the smallest deletion recovered leading to a change in eye phenotype was approximately 34kb (line 7-M30). Despite doubling the number of chromosomes screened, no smaller deletions were recovered. A small duplication of approximately 4kb adjacent to 39C-12, which successfully altered the eye phenotype from variegating to red, was recovered. The sites of these recombination events might reflect \( P \) element's
insertion bias for 5' regulatory regions (SPRADLING et al. 1995; TSUBOTA et al. 1985), and thus might define the limit of resolution achievable in this region of the genome.

1360 is associated with most but not all variegating reporters in the Hcf region.

Previous studies of P element insertion lines in the chromosomal region surrounding 39C-12 have correlated the presence of the repetitious element 1360 with heterochromatin formation and a variegating eye phenotype. In particular, it was found that the presence of 1360 within less than 10kb from an insertion site is generally correlated with a variegating eye phenotype, while a distance greater than 10kb from 1360 correlated with red eye color (SUN et al. 2004). This observation is supported by additional insertion events recovered in the region between nucleotide positions 300,000 and 500,000 on the D. melanogaster chromosome four; the variegating lines 7-M1399, 7-M1365, and 7-M484 are closer than 10kb to a 1360 element, while the red eye line 7-M201 is more than 10kb away from the closest 1360 element. The same pattern is observed for most duplications and deletions generated in this region; the eye phenotype for deletion/duplication lines without a 1360 element closer than 10kb is red (7-M30, 7-M972/7-M973/7-M1067, 7-M369, and 7-M1148; see Figure 2A and 2C, and Table 2).

One notable exception to this trend is line 7-M53. The genomic deletion characterized in this fly stock spans approximately 99kb (see Figure 2A) and removes several 1360 fragments found upstream of the original insertion site (nucleotide position 380,116). The recombination event puts the P element junction just distal to a pair of 1360 elements, eliminating these, and close to the 3' end of a Cr1a element (465bp remain of
this element). The 10kb region distal to the insertion site contains two small INE element fragments, as well as a fragment of an FB element. The new sequences brought into close contact with the P element - and the white gene it carries - contain no nearby fragments of 1360. Given the variegating eye color of 7-M53, this finding suggests that sequence features other than 1360 impact chromatin structure.

**Chromatin domains on the fourth chromosome near sv**

To determine if the findings from studies of the Hcf region can be generalized to other regions on the fourth chromosome, a new P element screen was designed to investigate a second region of chromosome four of *D. melanogaster* (for the transgene construct see Figure 1A). The chromosomal region selected was more distal from the centromere, and again, based on previous data, euchromatic and heterochromatic domains appeared to be adjacent to each other (see the transgene insertion map in Figure 2C). In addition, this region exhibited a noticeable paucity of 1360 elements, raising the expectation that additional elements functioning as initiators of heterochromatin could be identified here. The P element insertion of line 4-M1030, giving a red eye phenotype, is at position 119,408 (cytological position 102D4) within the coding region of the sv gene (*SUN et al.* 2004). The goal of this screen was to further delineate the boundary between heterochromatin and euchromatin that appears to occur close to this location. We again attempt to identify critical repetitious elements by investigating local transposition events or local genomic deletions/duplications. In this case, we focus on lines where the eye color phenotype changed from red to variegating.
The cross employed is shown in Figure 1C, and additional details can be found in the Methods section. In total, 3,300 single male crosses (cross 2 in Figure 1C) were screened for putative mobilization events, and 326 males with a variegating eye phenotype were recovered (transposition rate > 10%). 202 lines showed inheritance patterns consistent with \( P \) element insertions on a single chromosome. The remaining lines showed either no inheritance of the PEV phenotype, or a more complex inheritance pattern; thus, they were excluded from further analysis. The reporter in most of the lines recovered mapped to chromosome four (179 lines; 88.6%), the second highest number of insertions were found on chromosome three (18 lines; 8.9%). Only two lines mapped to chromosome two and three lines to the Y chromosome. The large number of lines mapping to the fourth chromosome was expected, as the experimental design preferentially selected for local events.

In total, 129 of the new lines, exhibiting a wide range of variegation levels (see Supplementary Figure 1) and having \( P \) element reporters mapping to \( D. \) melanogaster chromosome 4, were subjected to molecular characterization. The results are presented in graphical form in Figure 3 (see also Supplemental Table 2). Southern blot analysis revealed that a large portion of the lines with an altered eye phenotype have a damaged \( P \) element (20%, see Figure 3) and/or multiple copies of the \( P \) element (46%). The latter lines include two or more copies of the insert in a variety of arrangements. While a few lines were identified as tandem, head-to-head or tail-to-tail duplications, the majority of lines carrying multiple copies of the \( P \) element did not show easily interpreted restriction enzyme digestion patterns. A switch from red to a variegating eye as a consequence of
multiple P element insertions is not unexpected (DORER and HENIKOFF 1994); however, given the specific goals of our study, these lines were excluded from further analysis.

The remaining lines (~34%) passed our initial Southern blot screen to eliminate multiple insertions and damaged P elements and were investigated further (see Table 3 and Figure 2B). We found that eleven transposition events had resulted in complex rearrangements of chromosome four (labeled "complex rearrangement" in Figure 3). Three lines with variegating eye phenotypes originated from true transposition events or "jumps." In 18 lines, the switch in eye color was caused by P element induced recombination leading to the deletion or duplication of a limited adjacent genomic region (six mobilizations of the 3' end of the P element, 9 mobilizations of the 5' end). The remaining events producing a switch in eye color phenotype from red to variegating are a particularly interesting category, albeit not for our present goal. They represent events, which we designated as "phenotypic switch," where the eye color of the flies changed from red to variegating; but our limited molecular analysis failed to detect any alteration in the P element or the surrounding genomic region. Given the nature of the molecular analyses employed, it is impossible to determine what mechanism caused these events; likely possibilities include genetic alterations too small to detect by Southern blot (such as duplication of the P element ends), and alterations further distal from the insertion site. As these lines do not contribute to our understanding of the chromatin domains adjacent to sv, they were excluded from further analyses.
Maps showing the new P element insertion lines produced by our investigation of the sv genomic region are provided in Figures 2B and 2C. The red eye color line 4-M1030 (nucleotide position 1,119,408) is flanked by two reporter lines exhibiting PEV, 118E-9 (nucleotide position 1,071,991) and 5-M72 (nucleotide position 1,161,677), which delineate the potentially euchromatic domain defined by 4-M1030. The newly identified line 8-M1 (nucleotide position 1,072,020) is slightly closer to 4-M1030, decreasing the size of the potentially euchromatic domain to 47,388bp. This domain encompasses all of sv and is approximately half the size of the second smallest putative euchromatic domain, defined by 7-M201, delineated by 5-M29 and 7-M484/7-M1365 to 106,863bp. The two other transposition events recovered in this study provide information about the chromatin domain surrounding the CAPS gene in the case of line 8-M22, and about the region upstream of Glu-RA in case of 8-M294 (see Figure 2C). Both of these lines exhibit variegating eye color indicating they inserted in a heterochromatic domain. We note that the CAPS gene is actively transcribed in nervous system tissues (RENDEN et al. 2001) and in the eye imaginal disc (KLEBES et al. 2002).

Additional information about the chromatin domains surrounding the insertion site of 4-M1030 was gained from the analysis of the local genomic deletions/duplications generated by our efforts. The smallest deletion that generated a change in eye phenotype from red to variegated was identified by the insert 8-M175 (deletion of 36,381bp from 1,083,027 to 1,119,408). This deletion encompasses part of sv, activin-beta, and RfaBp. Given the preference of P elements to insert within 5' regulatory regions (SPRADLING et al. 1995; TSUBOTA et al. 1985), this is most likely the smallest deletion that can be easily
generated. While the 5' regulatory region of \( sv \) is closer, 10kb proximal of the 4-M1030 insertion site, it is likely part of the euchromatic domain identified by 4-M1030, as may be the adjacent \( activin-beta \) promoter. It is interesting to note that records from FlyBase show only two transgene insertions annotated in the 40kb region from 1,080,000 to 1,120,000, one \( PBac \) insertion at nucleotide position 1,085,600 (close to the 5'end of \( RfaBp \)), and one \( P \) insertion at position 1,105,069 (at the 5' end of \( activin-beta \)).

**1360 is not associated with variegating \( P \) element insertions in the \( sv \) region of chromosome four**

Because the eye color phenotype of \( P \) element reporter lines in the genomic region surrounding \( Hcf \) showed a correlation with the presence/absence of the 1360 repetitive element (Sun et al. 2004), we investigated the potential for such a relationship with the lines recovered in the vicinity of \( sv \) as well. The region surrounding \( sv \) in general has a low density of 1360 with no 1360 element closer than 50kb. The transgene of 4-M1030 (red eye color) is located within the \( sv \) gene. The 20kb region surrounding the transgene is rich in \( INE \) elements (13 fragments; all repeated element information given is based on FlyBase annotation). There are also three fragments of \( Isfun-1-Dfun-like \) and a fragment of \( INE-1-Dbuz-like \). (There is a 52bp remnant of a 1360 element, but previous analyses have excluded elements of less than 60bp due to the increased possibility of misclassification.) Thus, 4-M1030 follows the previously reported pattern of red eye color being associated with the absence of 1360. However, analysis of the reporter lines with insertions or deletions in the flanking sequences in the region between 1,000,000 and 1,200,000 reveals no association between the presence of 1360 and a variegating eye
phenotype, with all variegating transposition lines within this region more than 10kb away from a 1360 element (Figure 2B). All of our computational analyses are based on the published Drosophila genome sequence, thus leaving open the possibility of sequence polymorphisms in the 1360 distribution between the fly strain used in this screen and the sequenced strain of D. melanogaster. To rule out this possibility, we investigated a 10kb region adjacent to the insertion site (both proximal and distal) for three variegating lines (8-M1, 8-M175, and 8-M262). Using a PCR assay to generate DNA fragments of a predicted size, we confirmed the absence of additional 1360 elements (or any other elements) larger than 80bp (data not shown) in these regions.

While several of the variegating lines in the sv region of chromosome four lack 1360 elements in close proximity, there are a large number of other repetitive elements adjacent to these P element insertion sites. The insertion site of 8-M1 is surrounded by seven INE fragments, one fragment of Tc1, and one fragment of ISBu2-Dbuzz-like. 8-M167 and 8-M262 show a similar pattern in the distribution of transposable elements, while 8-M175 has two INE fragments and one S fragment in its vicinity. Table 4 contains a list of all repetitive elements found within 10kb of each variegating P element insertion that does not have nearby copies of 1360 (more than 30 kb distance from 1360 based on Figure 4). While there is no common element found adjacent to all lines - with the exception of INE-1 - all of these repetitious elements can be considered candidates to act as heterochromatin initiators. A minimal set would include Tc1, S, and F elements. INE-1 is not a considered a candidate per se, as it is conspicuously associated with both euchromatic and heterochromatic domains on the fourth chromosome.
Reporters close to 1360 usually exhibit a variegating phenotype, but 1360 is not associated with all variegating inserts on the fourth chromosome.

Given the contrasting findings between the two genomic regions investigated, we examined the correlation between the presence of 1360 and the PEV induced eye phenotype for all fourth chromosome P element insertion lines isolated in the screens described here as well as in previous screens carried out by our laboratory. A graphical representation of the results is shown in Figure 4A, where all P element reporters are plotted against their distance to the closest fragment of 1360 larger than 60bp. When the reporter lies within 10kb of a copy of 1360, in our data set, there is a 94% probability of observing a variegating phenotype (16 out of 17 cases). On the entire chromosome, we find a weak association between the distance of the P element from 1360 and the eye phenotype resulting from the P element transgene. However, this association is not statistically significant when compared to the null model (see analysis below). Specifically, the absence of 1360 does not correspond with red eye color in lines such as 8-M1 or others at the right end of the distribution shown in Figure 4A. This finding indicates that other sequence elements besides 1360 may also drive heterochromatin formation on the fourth chromosome.

The original study which identified 1360 as a potential target for heterochromatin formation included P element insertion lines carrying genomic deletions and duplications in the analysis (Sun et al. 2004). In fact, most lines were not simple P element insertions. Thus, we analyzed the data derived from the two screens described in this
manuscript as well as the data from Sun and colleagues to generate Figure 4B, which, like Figure 4A, shows the distance of $P$ element reporters from 1360 and their eye color phenotype. The results are similar to those shown in Figure 4A; while proximity to 1360 predicates a variegating phenotype, the absence of 1360 in close proximity to the $P$ element reporter does not necessarily lead to a red eye phenotype. In this case, the variegating and red insertion lines are more interdigitated than in the original study, which showed a greater propensity for lines with 1360 more distant from the $P$ element reporter to have a red eye phenotype (Sun et al. 2004). Our findings indicate that the strong correlation observed previously might be specific to the genomic region surrounding $Hcf$ and argues that other elements play a role in chromatin packaging as a whole.

**The presence of any single repeat element cannot serve as predictor for eye phenotype of $P$ element reporters.**

As the statistical significance of the presence of any particular repetitious element is difficult to assess from graphs as shown in Figure 4, we conducted a statistical analysis using the simple $P$ element insertions on chromosome four generated in our lab (39 lines total). Based on the FlyBase annotation (release 5.1), there are 66 natural transposon types represented on the fourth chromosome. For each $P$ element insertion site, the number of nucleotides occupied by a specific transposon type within a given window was calculated, as well as a total number of nucleotides occupied by any transposon. The mean number of nucleotides occupied by a transposon was compared between variegating and red insertion lines, and the significance level evaluated by a permutation
test. The window size was increased in 1kb steps from 1kb to 50kb. This analysis revealed that red inserts are surrounded by significantly more \textit{INE} and \textit{HB} element sequences for certain window sizes (\textit{HB} - window size 25-50kb, excluding 27kb, 33kb, 40kb, and 41kb; \textit{INE} - window size 19kb-20kb).

Because several of the \textit{P} element insertion lines uncovered in the various genetic screens are clustered at certain hotspots along the chromosome (see Figure 2C), we pruned the list of \textit{P} element insertions included in the analysis to insertions more than 10kb apart from each other (18 lines). This second analysis is less likely to be influenced by local biases in transposon distribution. The results of this analysis reveal that the \textit{ID} element is enriched in regions of variegating inserts for a number of window sizes, but none are statistically significant when corrected for multiple testing. These findings indicate that no single repetitious element can serve as predictor for the effects of chromatin structure on a \textit{P} element reporter gene in all regions of the fourth chromosome.

\textbf{Variegation of the \textit{P} element transgene is generally dependent on HP1 irrespective of the causative event}

The \textit{P} element screen examining the \textit{sv} genomic region resulted in several classes of variegating insertion lines, including those due to transposition, presence of multiple \textit{P} elements and genomic deletions/duplications. These lines vary widely in their level of variegation (including lines with pigment levels similar to a \textit{white} mutant as well as lines with pigment levels close to wildtype; see Supplementary Figure 1). In general, previously identified variegating \textit{P} element reporters have shown increased eye pigment
levels (suppression of variegation) in the presence of a mutant allele of \textit{Su(var)2-5}, the gene coding for HP1 (EISENBERG \textit{et al.} 1990). Eye pigment levels of \textit{P} element insertion lines were determined for both males and females carrying one copy of the \textit{P} element reporter either after a cross to \textit{yw}, or in the presence of the \textit{Su(var)2-5}^{02} allele. A subset of the results for males is shown in Figure 5 (additional data, see Supplemental Figure 1). While eye pigment levels are generally lower in females, the trends in females are similar to those seen males (see Supplemental Figure 1). The lines tested vary in the level of PEV they exhibit, which is reflected by the amount of eye pigment measured after a cross to the \textit{yw} stock (see Figure 5, yellow bars). The variegating phenotype is caused by different molecular events - 8-M1 by a transposition event; 8-M175, 8-M318, and 8-M262 by genomic deletions; 8-M94 by the presence of multiple \textit{P} elements; 8-M293 and 8-M11 by a "phenotypic switch" due to unknown reasons. Despite these differences, all reporters respond to the presence of the \textit{Su(var)2-5}^{02} allele with an increase in eye pigment levels, with many reaching 60-70\% of wildtype levels. A noticeable exception is 8-M11 (one of the lines classified as "phenotypic switch"). This line shows an increase in eye pigment in the presence of the \textit{Su(var)2-5}^{02} allele; however, even this increased pigment level is much lower than that of any other line, and resembles more closely the values observed in lines with strong PEV in the \textit{yw} stock (generally those with insertions of the reporter into regions of pericentric heterochromatin).
DISCUSSION

The goal of this study is to further our understanding of the chromatin domains composing the fourth chromosome of *D. melanogaster*. Previous studies based on PEV assays had indicated that on this small chromosome heterochromatic and euchromatic domains are interspersed (Sun et al. 2004). The data presented here confirm these results. In particular, we were able to confirm the domain structure near *Hcf*, where small genomic deletions or duplications are capable of altering the PEV phenotype (Sun et al. 2004). We have isolated a small duplication of 4 kb which is capable of altering the expression status of the *P* element reporter gene, evident in the change of eye color from variegating to red. This finding, combined with the previous finding that a 22 kb deletion can also cause a switch in phenotype, indicates that this region of chromosome four is capable of sustaining both heterochromatic and euchromatic structures within a 26 kb region.

Despite increasing the number of events screened more than two-fold, we were unable to isolate a deletion smaller than 22 kb in the *Hcf* region. This result might be a reflection of the limitations of the *P* element construct used. *P* elements show insertion bias, preferring the 5'-end of genes over other sites (Bellén et al. 2004; Spradling et al. 1995). In proximity to line 39C-12, the starting line for the screen, the closest 5'-untranslated region and gene is approximately 22 kb from the insertion site. Deletions and duplications in this experiment are most likely derived from *P* element induced recombination events [via Hybrid Element Insertion, (Preston and Engels 1996)], a process known to generate deletions as small as 10 bp (Preston and Engels 1996).
In the course of our investigation, we identified a new euchromatic domain on chromosome four. 7-M201, a line with red eye color, was used to characterize this new domain; its P element reporter is located at nucleotide position 436,566 in a region proximal to the lgs gene. This finding increases the total number of euchromatic domains on the fourth chromosome discovered by the PEV assay to four. The genomic region defined by 7-M201 is interesting, as it is within an approximately 50kb region proximal to lgs that is devoid of genes. Within this region, there are numerous repeated elements, but no annotated expressed sequences (based on FlyBase, annotation 5.1). In contrast, the P element of 4-M1030, which is located within the sv gene, identifies a euchromatic domain that is gene-rich while still containing numerous transposable elements. The euchromatic domain surrounding zfhh2 appears to have similar characteristics, as does the region distal to ci characterized by line 2-M2021. These data indicate that sequence contexts that appear very different on a cursory evaluation can nonetheless all sustain the formation of euchromatic structures.

Our investigation focusing on the sv region of chromosome four also confirmed our previous results. Similar to the results gained from the studies of the Hcf region, we find that P element induced genomic deletions and duplications can lead to altered eye phenotypes. While in this aspect the results from our studies of the Hcf and sv regions are similar, there are several significant differences. One primary difference is in the type of lines recovered. This difference is mainly due to the phenotype that was used for selection. In the study of the Hcf region, we selected for red eye color (or a
recombination event), and consequently, most lines retained single copy intact \textit{P} elements. In contrast, in the study of the \textit{sv} region, we selected for variegating eye color, and, not unexpectedly, recovered many lines that were neither transpositions, nor genomic deletions and duplications. The large number of \textit{P} element double insertions (resulting in variegation) recovered was noteworthy, especially since most of them were not simple tandem, head-to-head or tail-to-tail duplications. Previous work has demonstrated that variegating phenotypes can be observed due to repeat-induced gene silencing (RIGS) in Drosophila as well as in other species (HENIKOFF 1998; MEYER 1996). RIGS occurs when more than three copies of a \textit{P} element transgene are arrayed at a single genomic location irrespective of their relative orientation (DORER and HENIKOFF 1994). Silencing and PEV can also occur if there are only two copies; however, the mechanisms of silencing seem to differ as these latter lines do not respond to the presence of a mutant allele of \textit{Su(var)2-5} (DORER and HENIKOFF 1994) and may also differ in other aspects as well (JOSSE \textit{et al.} 2002). In our study the lines with multiple \textit{P} elements all showed a positive response to \textit{Su(var)2-5}. Interestingly, previous studies have suggested that certain single copy insertions on the fourth chromosome show sensitivity to the \textit{P} repressor (regulatory sequences encoded by the \textit{P} element that can repress \textit{P}-associated hybrid dysgenesis) although this usually only affects multiple copies subject to RIGS (JOSSE \textit{et al.} 2002).

We previously reported that \textit{P} element reporters exhibiting a variegating phenotype are associated with the \textit{1360} repeat element in the \textit{Hcf} region (SUN \textit{et al.} 2004). Studies here confirm that proximity to \textit{1360} is correlated with silencing, but show that a switch to a
variegating phenotype can occur in the absence of 1360. Specifically, analysis of sequences surrounding the sv gene found no association between the distance to 1360 and the P element induced eye phenotype. Our statistical analysis of the entire fourth chromosome confirmed this result and showed no significant correlation between the presence of any single repeated element and the P element's expression status. Genetic experiments indicate that the presence of 1360 does have an impact on the P element's expression state in sequence contexts other than the Hcf region originally studied (HAYNES et al. 2006). However, these genetic studies also show that the presence of a single copy of 1360 is insufficient to cause heterochromatin formation irrespective of genomic location. Most P element reporter lines inserted in euchromatin that carry a copy of 1360 in addition to hsp70-white exhibit red eye color (HAYNES et al. 2006). Thus both the specific element and the genomic context appear critical.

One limitation of the identification of heterochromatic domains by a PEV screen is that extremely heterochromatic domains, i.e. those silencing the white reporter completely, would remain undetected. Due to the expected high false positive rate (due to loss of or damage to the P element), white-eyed flies were not collected and assayed in this screen; instead, the presence of some red omatidia was required for individual males to be included in our assays. This screening method does identify variegating P element insertion lines with very low eye color levels, similar to those of white mutant individuals (see Supplemental Figure 1), indicating very effective silencing in these cases. Nonetheless, it remains possible that different rules apply to cases where silencing is
complete, and the fly has a white eye. Our analysis pertains to cases where the PEV phenotype is observed.

Overall, our different findings in the $Hcf$ and $sv$ regions suggest that formation of stable heterochromatin requires several conditions to be met. Sequence context clearly is important, despite the fact that no single transposable element has been identified that is responsible for the formation of all heterochromatin. The lack of a simple correlation between eye phenotype and repetitive element distribution can be explained in part by observations that while the likelihood of observing PEV for any given gene decreases with increased distance from heterochromatin, this process is by no means strictly linear and has been noted to skip genes (TALBERT and HENIKOFF 2000). If, for example, chromosome looping occurs, the effective distance of a repetitive element to a reporter gene is very different from the linear distance based on the DNA sequence alone. However, in the absence of data from 3C studies or similar approaches, meaningful computational analyses are restricted to testing linear models. A repetitive nature is commonly associated with heterochromatin, and repeats, such as the $P[lacZ]$ insert arrays that lead to the discovery of RIGS in Drosophila, can induce ectopic heterochromatic sites that recruit the proper chromatin components (FANTI et al. 1998). Thus it appears that numerous sequences can initiate the formation of heterochromatin structure, given the correct circumstances. Based on the data currently available, requirements for stable heterochromatin formation likely include a certain level of repetitiveness in the DNA sequence, specific nuclear localization on a three dimensional level (CRYDERMAN et al. ...)
1999), and recognition by a targeting mechanism that identifies specific sequence elements (including but not limited to 1360) using characteristics as yet unknown.
ACKNOWLEDGEMENTS

We would like to thank the following undergraduates who participated in the two screens for their hard work and dedication: Kwame Adu-Wusu, Adrian Baudy (IV), Kelli Grim, Gladys Mensah, Rachel Shevchek, Cory Simpson, and Miriam Wiegand. We would also like to thank Jessica Plante for technical assistance and members of the Elgin lab for critical reading of the manuscript and helpful discussions.

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Table 1. Screen summary

<table>
<thead>
<tr>
<th>Screen number</th>
<th>Starting lines; eye phenotype</th>
<th>Selected eye phenotype</th>
<th>No. of chromosomes screened</th>
<th>Total no. of lines recovered on chr. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>39C-12; variegated</td>
<td>red or variegated; spa</td>
<td>2873</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>4-M1030; red</td>
<td>variegated</td>
<td>3300</td>
<td>129</td>
</tr>
</tbody>
</table>
Table 2. $P$ element insertion lines recovered in the investigation of the $Hcf$ region.

Nucleotide positions are given with reference to release 5.1 of the $D. melanogaster$ genome sequence.

<table>
<thead>
<tr>
<th>Line</th>
<th>Eye color</th>
<th>P/P viable?</th>
<th>Event</th>
<th>Cytological position</th>
<th>Nucleotide position</th>
<th>Description of insertion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>39C-12</td>
<td>var</td>
<td>yes</td>
<td>jump from X chr.</td>
<td>102B5</td>
<td>380116</td>
<td>proximal of $Hcf$</td>
</tr>
<tr>
<td>7-M201</td>
<td>red</td>
<td>yes</td>
<td>jump</td>
<td>102B8</td>
<td>436566</td>
<td>proximal of $lgs$</td>
</tr>
<tr>
<td>7-M547</td>
<td>red</td>
<td>no</td>
<td>jump</td>
<td>102C3</td>
<td>528024</td>
<td>within $zfh2$</td>
</tr>
<tr>
<td>7-M1015</td>
<td>red</td>
<td>no</td>
<td>jump</td>
<td>102C3</td>
<td>527838</td>
<td>within $zfh2$</td>
</tr>
<tr>
<td>7-M30</td>
<td>red</td>
<td>no</td>
<td>deletion</td>
<td>414195</td>
<td>distal to $CG2052$</td>
<td></td>
</tr>
<tr>
<td>7-M972*</td>
<td>red</td>
<td>no</td>
<td>deletion</td>
<td>414209</td>
<td>distal to $CG2052$</td>
<td></td>
</tr>
<tr>
<td>7-M973*</td>
<td>red</td>
<td>no</td>
<td>deletion</td>
<td>414209</td>
<td>distal to $CG2052$</td>
<td></td>
</tr>
<tr>
<td>7-M1067*</td>
<td>red</td>
<td>no</td>
<td>deletion</td>
<td>414209</td>
<td>distal to $CG2052$</td>
<td></td>
</tr>
<tr>
<td>7-M369</td>
<td>red</td>
<td>yes</td>
<td>duplication</td>
<td>376009</td>
<td>within $CG2165$</td>
<td></td>
</tr>
<tr>
<td>7-M1148</td>
<td>red</td>
<td>yes</td>
<td>duplication</td>
<td>90334</td>
<td>within $pan$</td>
<td></td>
</tr>
<tr>
<td>7-M484</td>
<td>var</td>
<td>no</td>
<td>jump</td>
<td>102C1</td>
<td>487401</td>
<td>distal of $bip2$</td>
</tr>
<tr>
<td>7-M1061</td>
<td>var</td>
<td>yes</td>
<td>jump</td>
<td>unknown</td>
<td>within $1360$</td>
<td></td>
</tr>
<tr>
<td>7-M1365</td>
<td>var</td>
<td>no</td>
<td>jump</td>
<td>102C1</td>
<td>487401</td>
<td>distal of $bip2$</td>
</tr>
<tr>
<td>7-M1399</td>
<td>var</td>
<td>yes</td>
<td>jump</td>
<td>102B3</td>
<td>332186</td>
<td>proximal of $CG2177**$</td>
</tr>
<tr>
<td>7-M53</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>479355</td>
<td>proximal to $bip2$</td>
<td></td>
</tr>
<tr>
<td>7-M586</td>
<td>red</td>
<td>yes</td>
<td>duplication</td>
<td>n. d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-M1244</td>
<td>red</td>
<td>yes</td>
<td>duplication</td>
<td>n. d.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These lines were established from three siblings and most likely represent a single $P$ element induced recombination event.
** Based on inverse PCR results, the 7-M1399 insertion is located within a \textit{1360} element adjacent to the nucleotide position listed in the table. However, based on the published genome sequence, there is no \textit{1360} element at this location, indicating that there has been a transposition event of \textit{1360} in 7-M1399 or the starting line.

\textit{var} - variegated

\textit{n. d.} - not determined
Table 3. $P$ element insertion lines recovered in the investigation of the $sv$ region.

Nucleotide positions are given with reference to release 5.1 of the $D. melanogaster$ genome sequence. For several lines, viability of the homozygous $P$ element lines was not determined because these lines are lethal over the $ci^D$ fourth chromosome balancer; thus, they are maintained as the segregating population that resulted after the screen, and it is unknown if they are homozygous viable.

<table>
<thead>
<tr>
<th>Line</th>
<th>Eye color</th>
<th>P/P viable?</th>
<th>Event</th>
<th>Cytological position</th>
<th>Nucleotide position</th>
<th>Description of insertion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-M1030</td>
<td>red</td>
<td>yes</td>
<td>n. a.</td>
<td>102D4</td>
<td>1119408</td>
<td>within $sv$</td>
</tr>
<tr>
<td>8-M1</td>
<td>var</td>
<td>yes</td>
<td>jump</td>
<td>102D1-102D3</td>
<td>1072020</td>
<td>within $CaMKII$</td>
</tr>
<tr>
<td>8-M22</td>
<td>var</td>
<td>n. d.</td>
<td>jump</td>
<td>102E1</td>
<td>1260865</td>
<td>within $CAPS$</td>
</tr>
<tr>
<td>8-M294</td>
<td>var</td>
<td>n. d.</td>
<td>jump</td>
<td>102C5-102C6</td>
<td>955301</td>
<td>proximal of $Glu-RA$</td>
</tr>
<tr>
<td>8-M90**</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102C5</td>
<td>931047</td>
<td>downstream of $CG32021$</td>
</tr>
<tr>
<td>8-M91**</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102C5</td>
<td>931047</td>
<td>distal of $CG32021$</td>
</tr>
<tr>
<td>8-M167</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102D1</td>
<td>1052491</td>
<td>proximal of $ATPsyn-\beta$</td>
</tr>
<tr>
<td>8-M175</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102D3</td>
<td>1083027</td>
<td>distal of $Zyx102EF$</td>
</tr>
<tr>
<td>8-M192</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102C5</td>
<td>928687</td>
<td>within $CG32021$</td>
</tr>
<tr>
<td>8-M199</td>
<td>var</td>
<td>n. d.</td>
<td>deletion</td>
<td>n. d.</td>
<td>n. d.</td>
<td>within $1360$</td>
</tr>
<tr>
<td>8-M262</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102D1-102D3</td>
<td>1071672</td>
<td>within $CaMKII$</td>
</tr>
<tr>
<td>8-M316</td>
<td>var</td>
<td>yes</td>
<td>duplication</td>
<td>102E1</td>
<td>1261532</td>
<td>within $CAPS$</td>
</tr>
<tr>
<td>8-M318</td>
<td>var</td>
<td>no</td>
<td>deletion</td>
<td>102C5</td>
<td>933545</td>
<td>proximal of $eIF-4G$</td>
</tr>
<tr>
<td>8-M43*</td>
<td>var</td>
<td>no</td>
<td>complex rearrangement</td>
<td>102B3</td>
<td>327980</td>
<td>proximal of $Rad23$</td>
</tr>
<tr>
<td>8-M66*</td>
<td>var</td>
<td>no</td>
<td>complex</td>
<td>102B3</td>
<td>327980</td>
<td>proximal of $Rad23$</td>
</tr>
</tbody>
</table>
Due to its position within a 1360 element, the exact position of this insertion on the fourth chromosome is unknown.

These lines were established from siblings in each case and most likely represent a single P element induced recombination event.

<table>
<thead>
<tr>
<th>8-M235</th>
<th>var</th>
<th>n. d.</th>
<th>complex rearrangement</th>
<th>102C2</th>
<th>578182</th>
<th>proximal of Pur-α</th>
</tr>
</thead>
</table>

n. a. - not applicable
n. d. - not determined
Table 4. Repetitive elements found adjacent to variegating P element reporters independent of 1360. Shown are repetitive elements found in close proximity (less than 10 kb) to variegating P element reporters that can not be explained by 1360. Only the results for variegating lines more than 30kb distal to a known 1360 element are shown (see Figure 4 for details). Annotation based on release 5.1 of the Drosophila genome.

<table>
<thead>
<tr>
<th>Line (genomic region examined)</th>
<th>Transposable element present</th>
</tr>
</thead>
<tbody>
<tr>
<td>39C-28/39C-24 (1,042,339-1,064,486)</td>
<td>HB, INE-1, INE-1-Dbuz-like, ISBu2-Dbuz-like, Tc1</td>
</tr>
<tr>
<td>118E-9/8-M1 (1,061,991-1,082,020)</td>
<td>INE-1, ISBu2-Dbuz-like, S, Tc1</td>
</tr>
<tr>
<td>5-M263 (487,401-497,401)</td>
<td>F, FB, INE-1</td>
</tr>
<tr>
<td>7-M53 (479,355-489,355)</td>
<td>Cr1a, FB, INE-1</td>
</tr>
<tr>
<td>8-M235 (568,182-578,182)</td>
<td>INE-1</td>
</tr>
</tbody>
</table>
**Figure 1.** Experimental design of the *P* element insertion screens deployed to probe the genomic regions surrounding *Hcf* (B) and *sv* (C).

A. *P* element construct. Schematic representation of the *P* element construct used in both genetic screens described in this article. Arrow heads represent the inverted *P* element ends (3' of the *P* element is on the left, 5' of the *P* element is on the right). The vertically hatched area corresponds to the *hsp70* promoter, while the diagonally hatched area represents the *hsp26* promoter. The grey box underneath represents the probe used for Southern blot analysis, a unique fragment of plant DNA. Arrows indicate transcription start sites and direction of transcription. (For a more detail description of the construct, please see (Sun et al. 2004).

*S* - *SalI* site; *X* - *XbaI* site; *B* - *BglII* site.

B. Crossing scheme employed to study the *Hcf* region of chromosome four in *D. melanogaster*. *P* represents the *P* element in a new site on chromosome 4. For details, please see the Materials and Methods section.

C. Crossing scheme employed to investigate the *sv* region of chromosome four in *D. melanogaster*. For details, please see the Materials and Methods section.
Figure 2. Maps of chromosome 4 showing the $P$ element insertion lines recovered in genetic screens investigating the regions surrounding $Hcf$ and $sv$ on chromosome four.

A. Close-up map of the chromosomal region surrounding the 39C-12 insertion site showing local transposition, deletion and duplication lines recovered in the screen investigating $Hcf$. Scale in kb. Coding sequences of the genes are marked as grey boxes below the scale bar; the * indicates the gene $CaMKI$. Positions of repeated elements are indicated by black boxes, with each box corresponding to a single element annotated by FlyBase (release 5.1 of the $D. melanogaster$ genome) that is larger than 150 bp. 1360 elements are marked with taller blue boxes. Individual insertion, deletion and duplication lines are shown, the insertion site marked by triangles. The color of the triangle corresponds to the fly eye color. The dot on the triangle denotes the 5' end of the $P$ element.

B. Close-up map of the chromosomal region surrounding the 4-M1030 insertion site showing local transposition, deletion and duplication lines recovered in the screen focusing on the $sv$ region. The notation is the same as described in part A, with the exception that only repeats larger than 300 bp are shown due to limitations of scale.

C. Map of the entire chromosome 4 showing $P$ element insertion lines recovered in the screens described here as well as screens previously published. Previously published lines are shown in blue (solid blue triangle indicates solid red eye color; spotted triangle indicates variegating eye color), while the lines newly described in this study as well as their parent lines are shown in red. In addition, a new deletion line that affects a very large section of the chromosome is included on this map. The two regions marked by brackets above the map (A, B) are the $Hcf$ and $sv$ regions under study, shown in Figures
2A and 2B. Eye phenotype is a function of the flanking DNA and indicates that most, but not all, of the fourth chromosome is packaged as heterochromatin.
**Figure 3.** Classification of lines recovered in the investigation of the sv region of chromosome four. For each category, the percentage of lines in this category is given. A switch in eye phenotype from red to variegating can occur as a consequence of $P$ element duplication (or damage), which suggests these events as well as changes in flanking DNA can precipitate heterochromatin formation.
Figure 4. Relationship between eye phenotype and proximity to 1360

A. The distance (bp) from each P element reporter insertion site to the closest fragment of the 1360 repetitious element is plotted with the eye phenotype conveyed by the P element indicated by the color of the bar (stippled - variegating; red- red). The X-axis was set to -10,000 to show the phenotype of P element insertions within a 1360 element (distance 0). Only simple transposition events were used in this compilation.

B. Same as in A, but here all P element insertions lines, including genomic duplications and deletions, are shown. While reporters within 10kb of 1360 are almost always packaged as heterochromatin, those more distant may or may not be so packaged, arguing that other elements must play a role in these cases.
Figure 5. All variegating lines recovered exhibit sensitivity to mutations in HP1. Shown are eye pigment levels for seven different $P$ element insertion lines in a $yw$ background (yellow bars) compared to the presence of the $Su(var)2-5^{02}$ allele (blue bars). All data shown are from males. In all cases, mutation in HP1 results in a loss of silencing.