Efficient Recovery of Centric Heterochromatin *P*-Element Insertions in *Drosophila melanogaster*

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ABSTRACT

Approximately one-third of the human and *Drosophila melanogaster* genomes are heterochromatic, yet we know very little about the structure and function of this enigmatic component of eukaryotic genomes. To facilitate molecular and cytological analysis of heterochromatin we introduced a *yellow*$^+$ (y^+)-marked *P* element into centric heterochromatin by screening for variegated phenotypes, that is, mosaic gene inactivation. We recovered >110 *P* insertions with variegated *yellow* expression from ~3500 total mobilization events. FISH analysis of 71 of these insertions showed that 69 (97%) were in the centric heterochromatin, rather than telomeres or euchromatin. High-resolution banding analysis showed a wide but nonuniform distribution of insertions within centric heterochromatin; variegated insertions were predominantly recovered near regions of satellite DNA. We successfully used inverse PCR to clone and sequence the flanking DNA for ~63% of the insertions. BLAST analysis of the flanks demonstrated that either most of the variegated insertions could not be placed on the genomic scaffold, and thus may be inserted within novel DNA sequence, or that the flanking DNA hit multiple sites on the scaffold, due to insertions within different transposons. Taken together these data suggest that screening for *yellow* variegation is a very efficient method for recovering centric insertions and that a large-scale screen for variegated *yellow P* insertions will provide important tools for detailed analysis of centric heterochromatin structure and function.

ONE of the more puzzling components of eukaryotic genomes is the role of heterochromatin. It is cytologically dense, remains condensed throughout the cell cycle, replicates late in S phase, and has a large proportion of highly repetitive DNA (John 1988). Heterochromatin occupies approximately one-third of the *Drosophila melanogaster* genome (Adams *et al.* 2000) and contains few mutable genes and a high proportion of repetitive sequences (Gatti and Pimpinelli 1992). Nevertheless, heterochromatin houses many essential functions, including genes for ribosomal RNA, as well as genes required for viability and fertility (Gatti and Pimpinelli 1992). In addition, heterochromatin plays a key role in chromosome segregation, since it contains elements responsible for meiotic pairing, sister cohesion, and centromere function/kinetochore formation (Dernburg *et al.* 1996b; Karpen *et al.* 1996; Dej and Orr-Weaver 2000; Sullivan *et al.* 2001). Despite the challenges inherent to structural studies of repetitive DNA, limited regions of heterochromatin have been sequenced and/or mapped molecularly in Drosophila, Arabidopsis, and mammals (Karpen and Spradling 1990, 1992; Haaf and Willard 1992; Wevrick *et al.* 1992; Sun *et al.* 1997; Copenhaver *et al.* 1999; Horvath *et al.* 2000; Schueler *et al.* 2001). However, most of the heterochromatin in the human and Drosophila genome sequences has not been sequenced or even restriction mapped (Adams *et al.* 2000; Lander *et al.* 2001; Venter *et al.* 2001). To understand the organization of the entire genome and chromosomal functions, we must produce more complete heterochromatin maps and sequences.

P-transposable elements have proven to be extremely useful tools for studying the Drosophila genome. In addition to their ability to mutagenize genes (Spradling *et al.* 1999), *P*-element insertions provide single-copy molecular-genetic entry points for elucidating the structure and sequence of a region. This property is especially useful when studying regions rich in repeated DNA (Karpen and Spradling 1992; Zhang and Spradling 1994; Cryderman *et al.* 1998; Zhang and Stankiewicz 1998). Flanking DNA sequences can be determined by cloning or inverse PCR (Spradling *et al.* 1995), restriction maps can be constructed using the single-copy *P* element as a probe (Karpen and Spradling 1992; Tower *et al.* 1993; Zhang and Spradling 1993), and the insertion site within heterochromatin can be determined by *in situ* hybridization to mitotic chromosomes (Zhang and Spradling 1994). However, *P* elements tend to be recovered much more frequently in euchromatic rather than heterochromatic sites (Berg and Spradling 1991).

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Although *P*-element insertions into heterochromatin have been identified by mutating heterochromatic genes (Devlin *et al.* 1990a,b), other investigators have successfully identified heterochromatic insertions on the basis of silencing of marker genes present in the *P* element (Zhang and Spradling 1994; Roseman *et al.* 1995). Heterochromatin-induced gene silencing [position effect variegation (PEV)] occurs when normally euchromatic genes are juxtaposed with heterochromatin, due to insertion or chromosome rearrangements (reviewed in Weiler and Wakimoto 1995). PEV manifests as mosaic or variegated silencing, such that some cells express the genes while others do not. Three mechanisms have been documented for PEV: (1) heterochromatin-induced change in the chromatin structure of euchromatic loci (Wallrath and Elgin 1995), (2) sequestration from euchromatic transcriptional machinery (nuclear compartmentalization) (Csink and Henikoff 1996; Dernburg *et al.* 1996a; Donaldson and Karpen 1997; Gerasimova *et al.* 2000; Hari *et al.* 2001), and (3) underreplication of the euchromatic gene (Karpen and Spradling 1990; Lilly and Spradling 1996).

Previous screens for heterochromatic insertions relied on partial silencing of the *rosy* (*ry*) eye-color gene, or the variegated expression of the *white* (*w*) eye-color gene (Karpen and Spradling 1992; Wallrath and Elgin 1995; Cryderman *et al.* 1998). These studies, although successful in obtaining heterochromatic insertions, did not produce enough insertions to allow broad dissection of the structure and function of the centric heterochromatin. Heterochromatin constitutes ~30% of the Drosophila genome, but only 1–3% of all insertions identified in these studies were located in the centric heterochromatin. The inability to recover a proportional number of centric insertions could be due to fewer insertion events, perhaps caused by lower accessibility of heterochromatic DNA during transposition. It is also possible that recovery of heterochromatic insertions is disfavored due to extreme repression of marker genes. The recovery of ry^+ insertions in centric heterochromatin by screening in the presence of a suppressor of variegation supports this hypothesis (Zhang and Spradling 1994).

One strategy for efficient recovery of centric heterochromatin insertions is to partially ameliorate marker gene repression by choosing a gene with a strong promoter. Previous investigations have shown that the body pigment gene *yellow* (*y*) is expressed in a variegated pattern when present in or near centric heterochromatin (Le *et al.* 1995; Murphy and Karpen 1995; Roseman *et al.* 1995). Could *yellow* be a stronger expressing gene when inserted in centric heterochromatin and thus lead to the recovery of a greater number of centric insertions? We previously used a y^+-marked *P* element to screen for mutants that would have a dominant effect on the inheritance of a "sensitized" minichromosome (Dobie *et al.* 2001) and simultaneously recovered insertions that variegated for *y*. Here, we report cytological and molecular analysis of the variegating insertions and demonstrate that centric heterochromatin *P*-element insertions can be recovered at high efficiency by selecting for variegating *y* insertions. We propose that the generation of a large collection of centric heterochromatin *P* insertions, on the basis of the methodology described here, will facilitate genomic analysis of the structure, sequence, and function of heterochromatin.

MATERIALS AND METHODS

Drosophila stocks, culture, and screen: Stocks and screening crosses were described previously (Dobie *et al.* 2001). Flies were grown on standard cornmeal/molasses/agar media (Ashburner 1990) at 25°. The letters in the line designations refer to different rounds of screening, and the numbers refer to insertions recovered in each round. Both letters and numbers indicate chronological order of recovery.

Analysis of variegation levels: Variegating males were crossed to $y^1\ w^{1118}$; ry^{506} virgins. Males heterozygous for the variegating insertion were analyzed visually for the level of w^+ expression in the eye and y^+ expression in the abdomen.

Cytological analysis of *P* insertions: Mitotic chromosomes were prepared from larval neuroblasts as described previously (Sun *et al.* 1997). Probes corresponding to the entire *SUPor-P* plasmid (Roseman *et al.* 1995) were labeled as previously described (Sun *et al.* 1997) and hybridized to squashes according to standard protocols (Sullivan and Warburton 1999). On some occasions, slides were denatured using 0.07 N sodium hydroxide to improve chromosome morphology. Images were captured as described previously (Sun *et al.* 1997). Centric insertions were those that could be localized to the cytological map of Drosophila heterochromatin (Gatti *et al.* 1994). Insertions at chromosome tips were deemed to be telomeric. The location of the *P* elements relative to the 4′,6-diamidino-2-phenylindole (DAPI) banding pattern on the heterochromatic map was determined by visual analysis in Photoshop (Adobe) and by independent quantitative analysis using IP Labs (Signal Analytics, Vienna, VA) and a fluorescence quantitation script (written by Beth Sullivan). A minimum of 10 prometaphase chromosomes were analyzed for each insertion in the high-resolution analysis.

Flanking sequence isolation and analysis: Genomic DNA was prepared as described previously (Eggert *et al.* 1998). Two to three flies worth of DNA was digested using 20 units of either *Hha*I or *Hpa*II (New England Biolabs, Beverly, MA) for 3 hr and then heat inactivated at 65° for 20 min. DNA equivalent to approximately one-fifth of a fly was ligated in a 20-μl volume using the Rapid Ligation kit (Roche Molecular, Indianapolis) following the manufacturer's instructions. Inverse PCR and sequencing were performed as described previously (Dobie *et al.* 2001).

BLASTN *vs.* nonredundant (nr) database analysis was performed on flanking sequences of >25 bp to identify homologous sequences in the Drosophila genome, expressed sequence tag (EST), transposable element, and repeat sequence databases (Berkeley *Drosophila* Genome Project 2001). Matches against the genome were determined by >95% sequence identity over >95% of the flanking sequence. Potential matches with ESTs were identified by a BLAST score >1000.

RESULTS

A screen designed to recover *P* insertions in centric heterochromatin: To identify genes that affect chromosome inheritance, we performed a screen for dominant

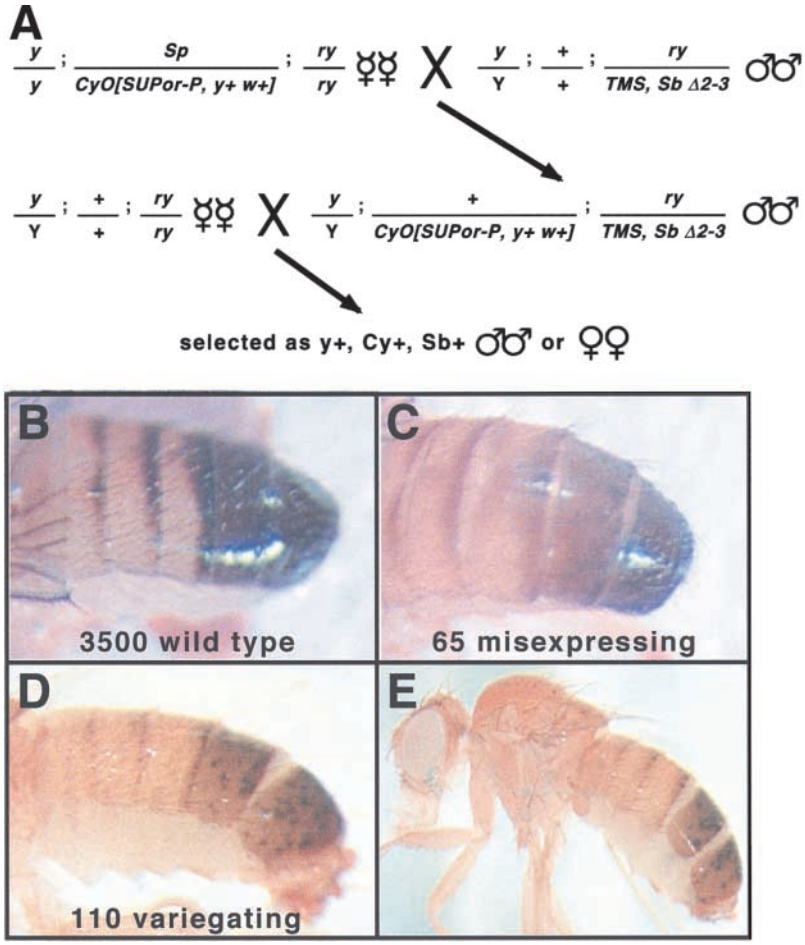

FIGURE 1.—Screen for heterochromatic *P* insertions. (A) *SUPor-P* was mobilized from position 60F on the CyO balancer. Mobilization in males allowed recovery of insertions in all chromosomes, including the Y, which is entirely heterochromatic. Mobilization events were recovered as y^+, straight-winged, and normal bristled males and females. $y = y^1$, $ry = ry^{506}$. (B) Wild-type male abdomen. (C) Misexpressing male abdomen. (D) Variegated male abdomen. (E) Very poor expression of *white*, with moderate variegation of *yellow*.

effects on sensitized minichromosome transmission (DOBIE *et al.* 2001), in which the *SUPor-P* insertion located at polytene chromosome position 60F was mobilized off the *CyO* balancer chromosome in males (Figure 1A). *SUPor-P* carries two reporter genes, y^+ and w^+, as well as two "suppressor of hairy wing" [Su(Hw)] binding sites flanking the w^+ gene (ROSEMAN *et al.* 1995). These binding sites increase the mutagenic properties of *SUPor-P* and also insulate w^+ from position effects (ROSEMAN *et al.* 1995). Both reporter genes on the *SUPor-P* element, *y* and *w*, have been shown to exhibit a variegated phenotype when put in close proximity to heterochromatin (ROSEMAN *et al.* 1995). Of ~3500 independent events recovered in our screen, 175 (5%) demonstrated two types of aberrant expression of *yellow*. The first group, referred to as "yellow misexpression" insertions (65 lines), displayed a general lightening of the pigmentation in the wings and/or the abdomen (Figure 1C), compared to wild type (Figure 1B). The second type, the "yellow variegators" (110 lines, 3.1%), exhibited predominantly *y* abdomens with y^+ spots or predominantly y^+ abdomens with patches of *y* pigmentation (Figure 1D).

Seventy-three *y* variegated insertions and 29 *y* misexpression insertions (102 total) were localized to specific parts of the genome by fluorescent *in situ* hybridization (FISH). FISH analysis was performed on neuroblast mitotic chromosomes, due to the underreplication and poor morphology of heterochromatin in polytene chromosomes (MIKLOS and COTSELL 1990), which were used in most previous localizations of heterochromatic insertions (ROSEMAN *et al.* 1995; WALLRATH and ELGIN 1995). The entire *SUPor-P* element was used as a probe, providing both a large region of homology for mapping the insertion (~11 kb), as well as internal positive controls; the *y* and *w* sequences hybridize to the tip of the X chromosome (Figure 2, A–F). Seventy-one (70%) of the insertions were in centric regions, 6 (6%) were telomeric, and 25 (25%) localized to the euchromatic arms of the X, second, or third chromosomes (Table 1). Unlike other screens (ROSEMAN *et al.* 1995; WALLRATH and ELGIN 1995), no insertions were found on the fourth chromosome (see DISCUSSION).

FISH localization demonstrates that selecting for variegating phenotypes, rather than misexpression of *y*, is a far more efficient way to isolate centric insertions. Strikingly, all 25 euchromatic insertions displayed the misexpression phenotype, rather than variegation (Table 1). The remaining four misexpression lines were telomeric. We conclude that none of the 29 nonvariegating, *y* misexpression insertions were centric, and these lines were not analyzed further. In contrast, 71 of the

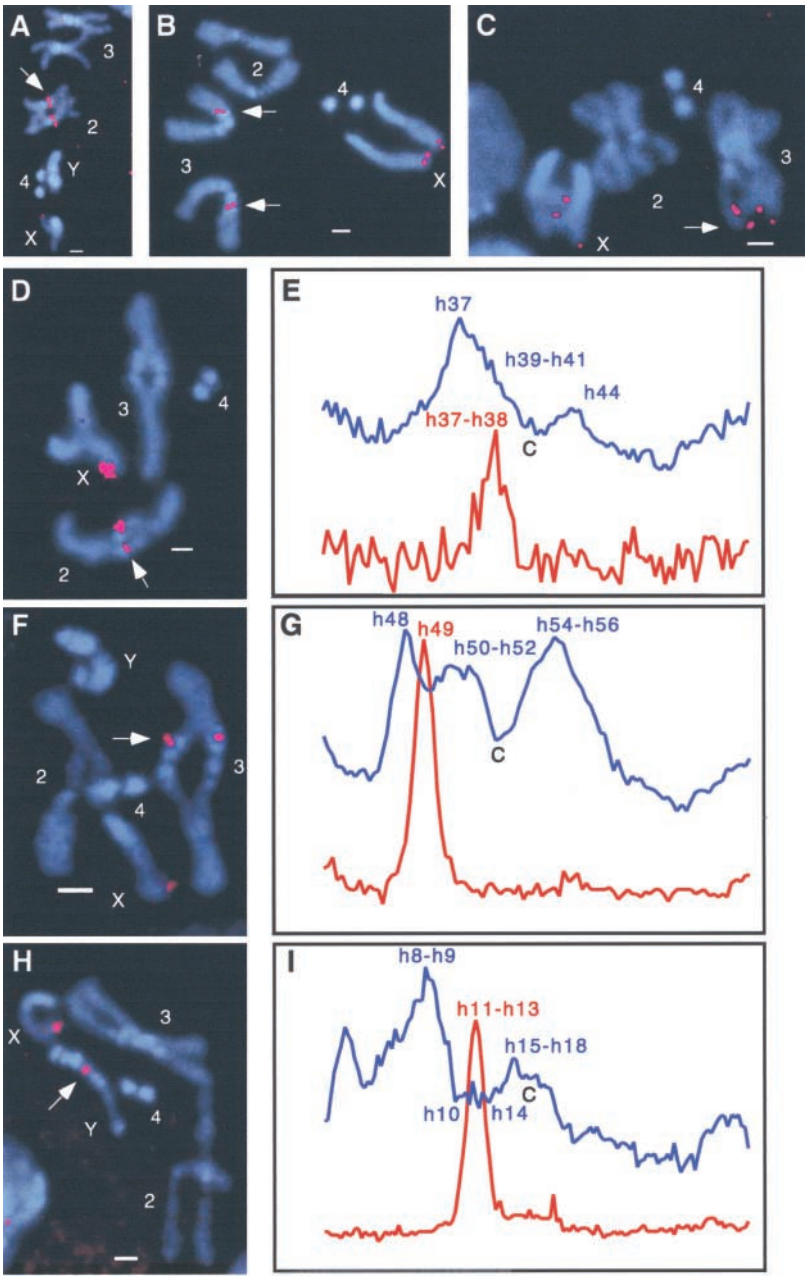

FIGURE 2.—FISH analysis of mitotic chromosomes. *P* elements were localized to metaphase chromosomes, using the entire *SUPor-P* as a probe. The tip of the X chromosome acts as a control for efficient hybridization. (A) Second chromosome centric insertion. (B) Third chromosome centric insertion. (C) Third chromosome telomeric insertion. High-resolution analyses were performed primarily on prometaphase chromosomes, which tended to have a lack of synapsis between heterochromatic regions (SMARAGDOV *et al.* 1980). (D and E) Second chromosome centric insertion into h37–38 and intensity graph. (F and G) Third chromosome centric insertion into h49 and intensity graph. (H and I) Y chromosome centric insertion into h11–h13 and intensity graph. Red, *SUPor-P* probe; blue, DAPI; C, centromere/primary constriction. Bars, 2 μm.

73 variegating insertions (97%) were in the centric heterochromatin; the remaining 2 insertions (3%) were telomeric (Table 1). Eighteen insertions (25% of 73 total variegators) were recovered on the Y chromosome, 17 (23%) were centric, and only 1 (1%) was telomeric. There were 29 insertions (40% of all variegators) on the second chromosome, and all were centric. The third chromosome had 25 insertions (34%); 24 were centric (33%) and only 1 was telomeric (1%). There was also a variegating insertion into the centric region of A887, which cytologically appeared to be a *de novo* minichromosome, presumably generated in the screen. We failed to recover variegated insertions on the X and fourth chromosomes, which are discussed later.

To determine if expression of marker genes plays a role in the recovery of centric insertions, a subset of variegated lines were crossed into a *y w* background to examine both *y* and *w* expression in males heterozygous for the *P* insertion. Both telomeric lines were examined, as well as five centric insertions from each of the second, third, and Y chromosomes. For all centric insertions, *y* expression was more easily visible; in fact, expression of *y* could be observed easily in several lines where *w* could not be detected at all (Figure 1E). The reverse was true for the third chromosome telomeric insertion; *w* expression was more visible than *y* expression (data not shown), consistent with previous observations (ROSEMAN *et al.* 1995).

Localization of centric *P*-element insertions to specific cytogenetic sites in heterochromatin: We performed

TABLE 1

Summary of insert distribution determined by metaphase FISH

	No. of insertions (% of insertions)						
Chromosome:	X	Y	2	3	4	A887	Total
Misexpression							
Centric	0	0	0	0	0	0	0
Telomeric	0	0	2 (8)	2 (8)	0	0	4 (14)
Euchromatic	3 (12)	—	12 (46)	10 (38)	0	0	25 (86)
Variegation							
Centric	0	17 (23)	29 (40)	24 (33)	0	1 (1.4)	71 (97)
Telomeric	0	1 (1)	0	1 (1)	0	0	2 (3)
Euchromatic	0	—	0	0	0	0	0

higher-resolution FISH analysis to determine the distribution of insertion sites. Prometaphase neuroblast chromosomes from 64 of the 71 nonminichromosome centric and Y chromosome insertions were localized with respect to the 61 heterochromatic cytogenetic bands (h1–h61; Gatti *et al.* 1994). Localization was quantitated by drawing a line through the axis of the chromosome and measuring the fluorescence of the FISH probe relative to the DAPI banding pattern (Figure 2, D–I). In some cases, insertion sites could not be localized to a single band and instead were localized conservatively to be within adjacent cytogenetic bands. Additionally, we were unable to resolve some adjacent bands, *e.g.*, h11–h13.

The high-resolution FISH analysis demonstrated that variegating insertions were present in slightly over one-third of the centric bands (23/61; Table 2, Figure 3). Considering that only 64 lines were localized, providing at best only onefold coverage of the 61 bands, this broad distribution suggests that recovery of *P*'s in most heterochromatic bands could result from generating more insertions. Nevertheless, the recovered insertions were clearly nonrandomly distributed within the centric heterochromatin. Insertions were recovered in all cytogenetic bands of the second chromosome except for h45. The third chromosome displayed a much less uniform distribution. The majority of third chromosome insertions, 19 of 21, inserted on the left arm from h47 to h49, mostly in h47–48. Only two insertions were recovered in any other region of chromosome 3 heterochromatin, in h57 and h58. Of the Y insertions, only one insertion was in the DAPI bright (AT-rich) regions of the long arm; one-half of the insertions (9 of 18) were located in h10–h14. Interestingly, three insertions were recovered in close proximity to the primary constrictions/centromeres of the second and Y chromosomes. In general, the insertions appeared to be concentrated in regions that contain previously mapped satellite DNAs (Lohe *et al.* 1993; Pimpinelli *et al.* 1995; Figure 3, see discussion).

In summary, the higher-resolution FISH analysis of prometaphase chromosomes demonstrated that the *P* insertions were broadly distributed within the centric heterochromatin, but "hot" and "cold" spots of insertion or recovery were also observed. More insertions must be generated to determine whether the coldspots are truly recalcitrant to insertion or recovery of *P* insertions and whether the hotspots are *bona fide* or represent statistical anomalies.

Sequence analysis of DNA adjacent to centric heterochromatin insertions: Variegated and silenced centric heterochromatin *P* elements have previously been demonstrated to insert adjacent to middle repetitive/transposon sequences (Zhang and Spradling 1995; Cryderman *et al.* 1998). Our FISH analysis demonstrated that many of the centric insertions recovered were in regions rich in satellite DNA, at the resolution of cytogenetic analysis (Figure 3). Inverse PCR was performed to directly examine the DNA sequences flanking the site of insertion (see materials and methods). We successfully amplified at least one 5′ or 3′ flank from 46 of 73 variegated insertions, including 44 out of 71 centric insertions (Table 2). This flanking DNA recovery frequency (63%) was significantly lower than the 98.9% frequency obtained in a recent analysis of euchromatic insertions (Liao *et al.* 2000). These results suggest that recovery of flanking DNA from heterochromatic insertions is less efficient than that for euchromatic insertions. Analysis of a larger collection of variegating *SUPor-P* insertions by the Berkeley *Drosophila* Genome Project (BDGP) produced flanking DNA from only 80% of the lines (A. Y. Konev, C. M. Yan, M. E. O'Hagan, R. A. Hoskins, G. Tsang, G. C. Liao, G. M. Rubin and G. H. Karpen, unpublished results), suggesting that the lower efficiency is not due to technical limitations.

For 43 of the 44 centric lines, >25 bp of 5′ and/or 3′ flanking DNA was recovered, allowing rigorous comparisons to sequences from the BDGP databases. The results are summarized in Table 2. Nineteen of the centric insertion flanks contained transposon sequences, including 1360, 1731, micropia, pilger, YOYO, hobo, and 297. One line (E760) was deep within the

TABLE 2

Insert locations and flanking sequences

	FISH location		Flanking sequences	
Line	Chromosome[a]	"h" band	Hits[b]	Genes
J564	YT	1–2	No hits	
C151	YC	3	AE002763 (unlocalized)	
B840.1	YC	10	Y-952	*indora*
F351	YC	10	No hits	
J448	YC	10	1360 and Su(Ste)	
J632.2	YC	10–13	AE003250 (unlocalized)	
B486	YC	14	AE003568 (X)	
C882	YC	11–13		
D425	YC	11–13		
I335	YC	11–13		
K013.1	YC	11–13	1360 and Su(Ste)	
B296	YC	16	Micropia	
D523	YC	17–18	No hits	
B783.2	YC	17–18		
D285	YC	20	No hits	
J718	YC	21		
B947	YC	22–24	1731	
E097	YC	22–24	TART	
C380	2C	35	AE003781 (2L)	*teashirt*
J730.1	2C	35		
C143*	2C	35–36	No hits	
K169	2C	35–36		
F443	2C	36		
B330	2C	36–37	AE003466	
G188.2	2C	37		
A338	2C	38		
C978.2	2C	38		
D459.2*	2C	38–41		
B554	2C	39–41	No hits	
C900	2C	39–41	Pilger	
D058	2C	39–41	1360	
E760	2C	39–41	Multiple	
F259	2C	39–41		
F702	2C	39–41		
G462.2	2C	39–41	1360 and stalker and Su(Ste)	
I997.2	2C	39–41	297	
J024	2C	39–41	1360	
K250.2	2C	39–41		
B841	2C	42–43		
C002	2C	42–43		
J209	2C	42–43	YOYO	
B720*	2C	42–46		
C657	2C	44		
J357	2C	44	AE002630 (2R)	
B879.1	2C	46	<25 bp	
C423	2C	46		
F806.2*	2C	Centric		
B283	3C	47	No hits	
H091	3C	47	AE002763 (unlocalized)	
J028.2	3C	47	1360 and Su(Ste)	
J985.2	3C	47	No hits	LD16643
K092	3C	47		
I019	3C	47–48		
I933.1	3C	47–48	1360 and Su(Ste)	
J322	3C	47–48	No hits	

(*continued*)

TABLE 2

(Continued)

Line	FISH location		Flanking sequences	
	Chromosome[a]	"h" band	Hits[b]	Genes
J685	3C	47–48	No hits	LD16643
B969*	3C	47–50		
B160	3C	48	No hits	LD16643
B168	3C	48	Could not be sequenced	
B319	3C	48	No hits	LD16643
C671	3C	48		
C907	3C	48	1731	
H127	3C	48	No hits	
J390	3C	48	No hits	
I202	3C	48–49	1360 and Su(Ste)	
C313.2*	3C	48–50	YOYO	
B898	3C	49	No hits	
J545	3C	49		
B415*	3C	49–51	1360 and Su(Ste)	
B079	3C	57	No hits	
B848	3C	58	Hobo	
J690.1	3T		HET-A	
A887	Mini			

* Lines in which high-resolution localizations did not meet the criteria for certainty (>10 chromosomes analyzed).

[a] C, centric; T, telomeric; mini, chromosome fragment that contains a *P* insertion.

[b] Failed inverse PCRs are indicated by blank fields in the "Hits" column. AE numbers refer to genomic scaffold contigs.

heterochromatin (h39–41) yet the flanking sequence hit the genomic scaffold multiple times; it shows no homology with known transposable elements and thus is likely to be a previously unidentified transposon or repeat. Seven insertions were unique hits on the genomic scaffold. H091 and C380 are located in the most distal heterochromatic bands of 2L and 3L, respectively, suggesting that the sequence scaffolds (Adams *et al.* 2000) extend into the heterochromatin in these regions. C380 is inserted near *l(2)04319*, a lethal PZ insertion in the *teashirt* gene (Spradling *et al.* 1999). The remaining five unique hits are located in normal euchromatic regions, yet FISH localization places the *P* insertion far from the ends of the euchromatic scaffolds. This suggests that there are unique regions in euchromatin that share significant homology to sequences deep within the centric heterochromatin. The remaining 16 lines failed to show high homology to either transposable elements or the genomic scaffold and presumably represent novel heterochromatic sequences.

Previous studies failed to recover insertions in simple satellite DNA (Zhang and Spradling 1995; Cryderman *et al.* 1998). None of the flanking DNA sequences recovered in our study contained satellite DNA, despite FISH localization of many of these inserts to satellite-rich regions (Figure 3). Either we were unable to recover *SUPor-P* insertions directly in satellites or flanks from insertions in satellite DNA could not be generated using inverse PCR. The lines we did not recover flanking DNA from (37%) may represent insertions in satellite DNA, as suggested by the FISH analysis. However, they could also have inserted in complex DNA, where no compatible restriction sites in the flanks correspond to the two enzymes used in the assay. Determining whether or not these insertions are within satellite DNA requires additional investigations.

Previous studies have identified *P* insertions in known heterochromatic genes (Devlin *et al.* 1990a,b; Zhang and Spradling 1994). To determine if any of the *y* variegators inserted in heterochromatic genes, we conducted a BLASTN analysis against the EST library for all nontransposon insertions (Table 2). Four of the lines (J985.2, J685, B160, and B319) had strong homology to an uncharacterized EST (LD16643). One line, B840.1, inserted in a region that showed high homology to the transcript of a gene *indora* (Rubin *et al.* 2000), a female-specific germ-line gene located on the second chromosome (Mukai *et al.* 1999). In summary, five lines corresponding to two loci had insertions in or near expressed heterochromatic sequences. However, this is likely to be an underestimate of the effectiveness of *SUPor-P* in mutating heterochromatic genes, since only approximately one-third (~5000) of the predicted Drosophila genes (~14,000; Adams *et al.* 2000) are represented

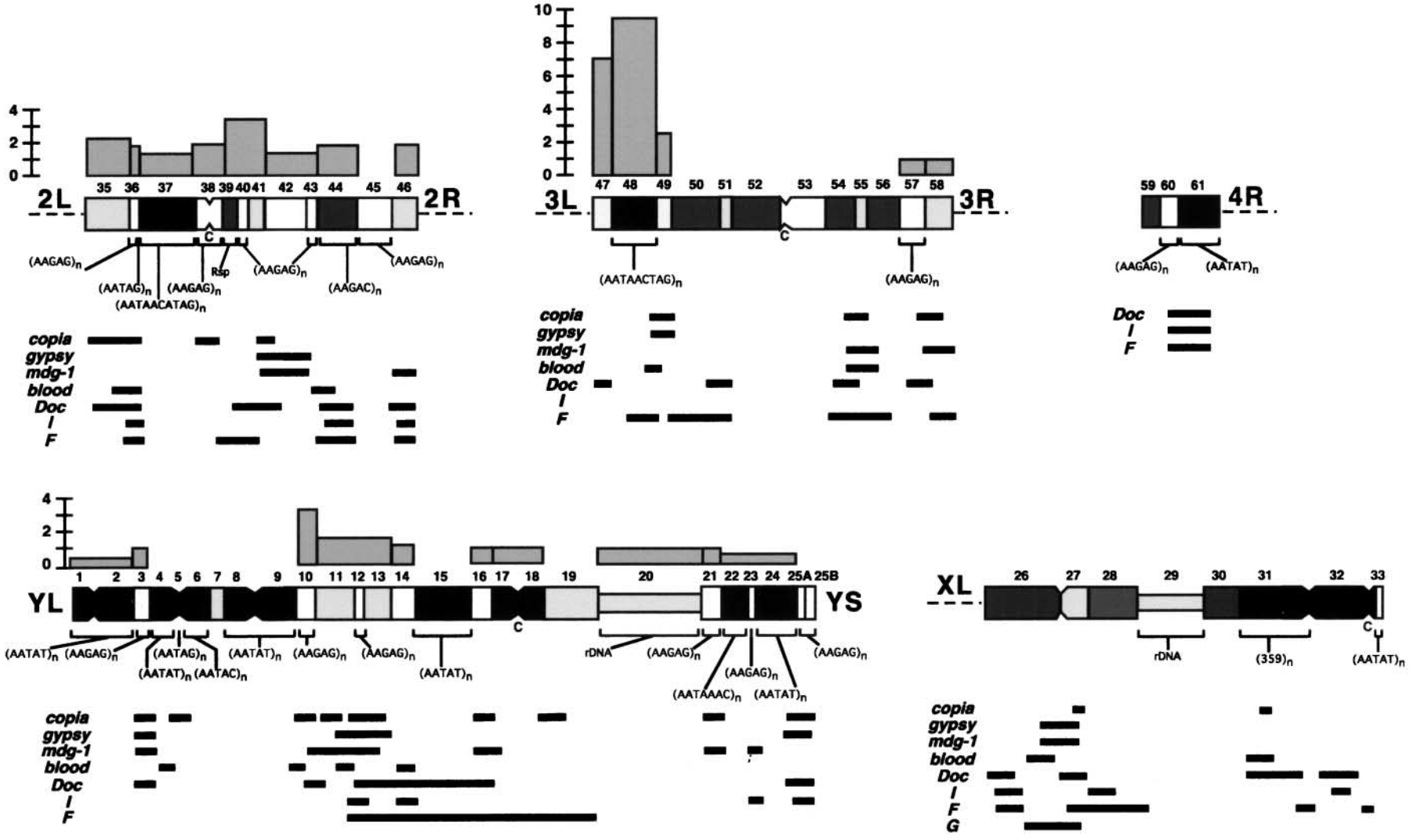

FIGURE 3.—Distribution of *P* insertions in Drosophila heterochromatin. The blocks represent the 61 cytogenetic bands of heterochromatin, and the dashed lines represent the euchromatic arms (GATTI *et al.* 1994). The DAPI intensity corresponds to the intensity of each block (the darker the block, the brighter the DAPI intensity). The distribution of satellite repeats as mapped by LOHE *et al.* (1993) and the distribution of transposons as mapped by PIMPINELLI *et al.* (1995) are indicated below the chromosomes. Bars above each chromosome represent the number of *P* insertions in that region, normalized according to the preciseness of the localization. Specifically, some *P* insertions could be localized to only two to four adjacent regions, depending on the resolution of the FISH signal and the DAPI banding pattern (Table 2). Assigning a value of 1 to each band in these cases would overstate the number of insertions that were localized specifically to each band. Therefore, localizations were divided among the relevant bands; *e.g.*, for an insertion that localized to 2 bands, each band was assigned a value of 0.5. To determine the number of total bands hit (23/61), insertions that localized to multiple bands were counted once. See Table 2 for the actual number of insertions that localized to different regions. Insertions marked with * in Table 2 are not included in this figure.

TABLE 3

Efficiency of screens in generating centric insertions

Marker	Variegated/silenced: % of total	% of variegated/silenced			Centric: % of total	Reference[a]
		Centric	Telomeric	Other location		
w and *y*	9.5	33	55	12	3.2	a
w	1.1	15	29	54	0.2	b
ry	4.0	87[b]	13[b]	0	3.5	c
y	3.1	97	3	0	3.0	This study

The screen using both *y* and *w* was performed using boundary elements flanking *w* (Roseman *et al.* 1995). The screen using *w* alone was performed without the benefit of boundary elements or suppressors of variegation (Wallrath and Elgin 1995; Cryderman *et al.* 1998). The screen using *ry* was performed in the presence of a suppressor of variegation (Zhang and Spradling 1994).

[a] a, Roseman *et al.* (1995); b, Wallrath and Elgin (1995) and Cryderman *et al.* (1998); c, Zhang and Spradling (1994).

[b] The Zhang and Spradling (1994) study produced four insertions in regions h1–2, which are reported here as telomeric, to be consistent with the criteria used in this study.

in the current EST database (Berkeley *Drosophila* Genome Project 2001).

DISCUSSION

Screening for *yellow* variegation yields a high frequency of centric heterochromatin insertions: The isolation and analysis of a large collection of *P*-element insertions into centric heterochromatin would greatly facilitate sequence, structural, and functional analysis of this difficult region of higher eukaryotic genomes. Previous studies identified heterochromatic insertions on the basis of insertion into a gene (Devlin *et al.* 1990a,b) or silencing of the *ry* or *w* marker genes (Zhang and Spradling 1994; Roseman *et al.* 1995; Wallrath and Elgin 1995). Our results demonstrate proof of the principle that high efficiency recovery of centric heterochromatin *P* elements results from scoring for *y* variegation of *SUPor-P* insertions (Table 3). This screen generated a large number of variegating lines (3.1% of all insertions), 97% of which were centric (71 of the 73 analyzed), rather than telomeric or euchromatic. High-resolution FISH mapping demonstrated recovery of insertions in 23 of the 61 cytogenetic bands, despite localizing only 64 lines (approximately onefold coverage of the 61 centric bands).

The overall frequency of centric insertion recovery (3%) was 17-fold higher than that in two previous studies, which used only variegating *w* as the genetic marker for heterochromatic insertions (Wallrath and Elgin 1995; Cryderman *et al.* 1998; Table 3). In a screen for *SUPor-P* lines that variegated for both *w* and *y*, 3.2% of the 349 total insertions (variegators and nonvariegators) were considered centric on the basis of hybridization to the chromocenter or to the base of an arm in polytene chromosome squashes, and an additional four variegators were mapped genetically to the Y chromosome. However, it is unclear how many of those insertions were truly within centric heterochromatin or inserted at the juxtaposition of heterochromatin and euchromatin, because none of the insertions were mapped by FISH to mitotic chromosomes. In addition, only 33% of insertions were centric, and 55% were telomeric (Roseman *et al.* 1995), significantly lower than the 97% proportion of centric insertions reported here (Table 3). Some of the *yellow* variegated events recovered in the Roseman *et al.* study may have been classified as misexpression in our screen, which would have increased the proportion of euchromatic and telomeric insertions. We conclude that screening for *y* variegation, as described here, is more efficient, because it requires performing cytological analysis on 3-fold fewer flies to recover the equivalent number of centric insertions.

Our overall frequency of centric insertions recovered from variegators (97%) is similar to a screen that used *ry* as the phenotypic marker (Zhang and Spradling 1994). However, obtaining this frequency of centric insertions required assessing the expression of *ry* in the presence and absence of an extra Y chromosome, to identify potential heterochromatic insertions that are silenced without the PEV suppressor. This approach is less efficient than screening for *y* variegation because it requires an extra generation to remove the extra Y. In addition, scoring for *ry* silencing (*ry* is nonautonomous and therefore does not produce variegated expression in the eye) is more difficult in comparison to *y* or *w* variegation.

The higher efficiency of centric insertion recovery compared to previous studies suggests that the use of *y* as a marker gene and focusing on lines with variegated phenotypes is a very effective method for recovering centric insertions (Table 3). Our expression studies revealed that insertions recovered by screening for *y* variegation often have poor or no expression of *w* (Figure 1). It is interesting that the *w* gene is surrounded by Su(Hw) insulator elements in the *SUPor-P*, and *y* is not,

yet *y* is less affected by centric silencing than *w*. The more robust expression of *y* likely facilitated the recovery of centric insertions that would be missed due to more complete silencing of the *w* gene. This conclusion is consistent with the fact that the previous *SUPor-P* screen yielded significantly more telomeric than centric insertions (Roseman *et al.* 1995); *white* expression is likely more visible in telomeric locations, where silencing is weaker. Similarly, the flanking sequence from one of our lines (B840.1) was identical to the flanking sequence from Y95-2 (Spradling *et al.* 1999), a *ry* Y insertion recovered by Zhang and Spradling (1993). Both insertions are in the same cytogenetic region (h10–h14), and the flanks are not homologous to any known transposon or repetitive element, suggesting that they are inserted in the same location. The insertions appear to be within 245 bp of each other, yet the Y95-2 ry^+ insertion is silenced completely without the addition of a PEV suppressor, whereas the variegating *y* gene in B840.1 is expressed at a level that allowed recovery in this screen without the presence of a PEV suppressor. This result supports the hypothesis that *y* is a more robust marker for centric insertions than *ry*, as we observed for *w*.

The distribution of centric insertions recovered in this screen was broad but nonrandom: FISH mapping the insertions to prometaphase chromosomes demonstrated that we recovered insertions in 23 out of a total of 61 heterochromatic bands. The fact that inserts were recovered in one-half the bands, despite only onefold coverage (64 centric insertions localized, 61 bands), demonstrates the utility of this approach to broad analysis of Drosophila centric heterochromatin. Nevertheless, there appear to be hotspots and coldspots for recovery of centric heterochromatin insertions, as observed for single *P* mutagenesis of the euchromatin (Spradling *et al.* 1999). For example, the Y chromosome contains over three times more centric heterochromatin than either the second or third chromosome (Peacock *et al.* 1978; Adams *et al.* 2000), yet we recovered more centric insertions on the second (28) and third (25) chromosomes than on the Y chromosome (17). Since most of the variegated insertions (>90%) were recovered as males (A. Y. Konev, C. M. Yan, M. E. O'Hagan, A. Lim, S. Tickoo, N. Vasquez, S. Kuman and G. H. Karpen, unpublished results) and only Cy^+ Sb^+ events were selected (the number of target chromosomes were equal), there should have been little overrepresentation of second and third chromosome insertions when compared to the Y. Preferential recovery of insertions in h47, h48, and perhaps h38–h41 suggests that these represent hotspots, and the absence of variegating insertions recovered on the fourth, the X, and most of the third centric heterochromatin suggests that these regions represent coldspots. However, definitive conclusions about recovery frequencies in different regions require generating more centric insertions than the onefold coverage reported here.

Regions that contain many or no recovered insertions may reflect differences in the ability of the *P* element to insert into different sites. Zhang and Spradling (1995) proposed that transposons and "complex islands" (Le *et al.* 1995; Sun *et al.* 1997) would be more receptive to *P*-element insertion in heterochromatin or that insertions in satellite DNA would not produce sufficient marker gene expression. An alternative hypothesis is that hotspots represent regions that are capable of inducing silencing, while coldspots represent sites that do not induce visible levels of silencing. We favor this hypothesis for the following reasons. Variegating insertions analyzed in this study localized to both the most DAPI-intense regions as well as the most DAPI-dull regions. Thus, AT composition does not appear to affect the capacity of heterochromatin to silence *y*. Additionally, there was no correlation between the distribution of insertions and the presence/absence of specific transposable elements in heterochromatin (Figure 3). BLAST analysis revealed that only 17 of 43 centric insertions were in known transposons. This suggests that the presence of nearby transposable elements and the AT composition do not affect silencing. The distribution of variegating insertions does correlate with the distribution of satellite DNA blocks. All variegating third chromosome insertions recovered in this study localized to the only two regions rich in known satellite repeats, and most variegating insertions were recovered in the second and Y centric heterochromatin, which have very high concentrations of satellite DNA (Figure 3). Previous localizations of silenced *ry P* elements also revealed clustering around regions rich in satellite DNA (Zhang and Spradling 1994). It is likely that regions of satellite DNA are especially effective at inducing silencing (Tolchkov *et al.* 1997; Kurenova *et al.* 1998) and thus recovery of insertions as variegators. Although none of the flanking DNAs amplified in this study contained known satellite DNA, it is likely that insertions in complex DNA "islands" contained within large "seas" of satellite DNA (Le *et al.* 1995; Sun *et al.* 1997) would still be subject to strong silencing.

The lack of insertions recovered in the X centric heterochromatin is of particular interest. X centric heterochromatin insertions were also not recovered in some previous screens in which the X chromosome was a potential target (Zhang and Spradling 1994; Roseman *et al.* 1995; Wallrath and Elgin 1995; Cryderman *et al.* 1998), suggesting that choice of *P* construct and starting site are not responsible for this deficiency. It is unlikely that X insertions would not variegate, since studies have shown that both *w* and *y* can variegate when brought into close proximity to the X centric heterochromatin due to chromosome rearrangements (Karpen and Spradling 1990; Weiler and Wakimoto 1995; FlyBase 2001) or by *P*-element insertion (Wall-

rath *et al.* 1996). The inability to recover X chromosome insertions could be due to the fact that mobilizations were generated exclusively in males. Under our mobilization scheme, X chromosome insertions would have been recovered in female progeny only, and we observed that variegated insertions were more frequently recovered in males than in females (A. Y. Konev, C. M. Yan, M. E. O'Hagan, A. Lim, S. Tickoo, N. Vasquez, S. Kuman and G. H. Karpen, unpublished results). Furthermore, subsequent analysis indicated that, in general, males carrying the insertion were more easily identified than females (data not shown). It is also possible that the unusual behavior of the X in spermatogenesis is responsible for the failure to recover X insertions. The failure to recover fertile X-autosome reciprocal translocations has been proposed to result from silencing of the X during spermatogenesis (Lifschytz and Lindsley 1972, 1974); such an altered chromatin state could also make centric heterochromatin recalcitrant to *P*-element insertion. We suspect that mobilization in females and recovery of variegators in males would overcome these problems and result in the recovery of X centric insertions.

Previous studies using *w* (Roseman *et al.* 1995; Wallrath and Elgin 1995), including one that utilized the same *SUPor-P* element and starting site described here, demonstrated that variegating insertions can be recovered in the fourth chromosome. We probably did not recover fourth chromosome insertions because the fourth is only 8% of the total centric heterochromatin of females and 7% of males (Peacock *et al.* 1978; Adams *et al.* 2000), and we recovered and mapped only 64 variegated centric insertions, which is far from saturation. It is also possible that the strength of the *yellow* expression in *SUPor-P*, combined with a weak silencing ability of the fourth, caused such insertions to be missed.

The use of *P* elements as single-copy entry points into heterochromatin structure, sequence, and function: The recent sequencing of the Drosophila and human genomes has left heterochromatin largely untouched (Adams *et al.* 2000; Lander *et al.* 2001; Venter *et al.* 2001). Of the 70 centric and Y chromosome insertions described here, 64 were localized to 23 of the 61 cytogenetic bands of centric heterochromatin, including one Y chromosome telomeric insertion. Whether the remaining 37 bands can be targets for *P* insertion or recovery cannot be answered in this study. A focused effort to increase the yield of insertions and the spectrum of insertion sites, utilizing suppressors of variegation (Zhang and Spradling 1994) and recovering X chromosome insertions in males, should address this question.

Ultimately, significant information about the molecular organization, sequence composition, and function of centric heterochromatin can be obtained by saturation of heterochromatin with *P* elements, even if the distribution is nonrandom. *P* elements greatly facilitate the molecular and structural analysis of heterochromatin by introducing unique, single-copy tags into repetitive regions (Karpen and Spradling 1992; Zhang and Spradling 1994; Cryderman *et al.* 1998; Zhang and Stankiewicz 1998). Information about flanking sequences can be correlated with cytogenetic localizations and gross molecular analysis, such as pulsed field gel electrophoresis, to create a molecular map of the heterochromatin. In addition, we were able to recover insertions with flanking sequences homologous to unlocalized and unordered bacterial artificial chromosomes (BACs; data not shown), which could help localize BACs that previously could not be placed within the genomic scaffold due to their repetitive nature. Finally, a large collection of *P* insertions would greatly facilitate functional analysis of heterochromatin, for example, by identifying and mutating the estimated 250–500 heterochromatic genes (Devlin *et al.* 1990a,b; Zhang and Stankiewicz 1998; Adams *et al.* 2000). In addition, *P* insertions can be used to examine the behavior of specific regions of heterochromatin with respect to chromatin structure and PEV (Wallrath and Elgin 1995; Sun *et al.* 2000, 2001), the cell cycle and replication (Karpen and Spradling 1990, 1992; Zhang and Spradling 1995; Lilly and Spradling 1996; Leach *et al.* 2000), and the organization of the interphase nucleus (Csink and Henikoff 1996; Dernburg *et al.* 1996a; Gerasimova *et al.* 2000; Hari *et al.* 2001).

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