Genetics of *P*-Element Transposition Into *Drosophila melanogaster* Centric Heterochromatin

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ABSTRACT

Heterochromatin is a major component of higher eukaryotic genomes, but progress in understanding the molecular structure and composition of heterochromatin has lagged behind the production of relatively complete euchromatic genome sequences. The introduction of single-copy molecular-genetic entry points can greatly facilitate structure and sequence analysis of heterochromatic regions that are rich in repeated DNA. In this study, we report the isolation of 502 new *P*-element insertions into *Drosophila melanogaster* centric heterochromatin, generated in nine different genetic screens that relied on mosaic silencing (position-effect variegation, or PEV) of the *yellow* gene present in the transposon. The highest frequencies of recovery of variegating insertions were observed when centric insertions were used as the source for mobilization. We propose that the increased recovery of variegating insertions from heterochromatic starting sites may result from the physical proximity of different heterochromatic regions in germline nuclei or from the association of mobilizing elements with heterochromatin proteins. High frequencies of variegating insertions were also recovered when a potent suppressor of PEV (an extra *Y* chromosome) was present in both the mobilization and selection generations, presumably due to the effects of chromatin structure on *P*-element mobilization, insertion, and phenotypic selection. Finally, fewer variegating insertions were recovered after mobilization in females, in comparison to males, which may reflect differences in heterochromatin structure in the female and male germlines. FISH localization of a subset of the insertions confirmed that 98% of the variegating lines contain heterochromatic insertions and that these schemes produce a broader distribution of insertion sites. The results of these schemes have identified the most efficient methods for generating centric heterochromatin *P* insertions. In addition, the large collection of insertions produced by these screens provides molecular-genetic entry points for mapping, sequencing, and functional analysis of Drosophila heterochromatin.

THE division of chromosomes into euchromatic and heterochromatic regions is perhaps the most striking and enigmatic aspect of genome organization in multicellular eukaryotes. Heterochromatin was originally defined as differentially staining regions of chromosomes, which retained a compact appearance throughout the cell cycle (Heitz 1928). Other unusual characteristics include late replication, regular nucleosome spacing, relatively inaccessible chromatin, the ability to silence euchromatic genes, and positioning in a distinct subnuclear domain at the periphery of interphase nuclei (reviewed in John 1988; Zhimulev 1998; Hennig 1999; Gasser and Cockell 2001; Grewal and Elgin 2002). Heterochromatin is a major component of higher eukaryotic genomes, comprising ~30% of both the fly and human genomes. It is concentrated in large blocks in the centric and subtelomeric regions of all chromosomes and is composed of highly repeated short sequences (satellite DNAs), middle-repetitive elements (predominantly transposable elements), and some single-copy DNA and genes. Despite an abundance of repetitive DNAs, heterochromatin is not functionally inert. It harbors the ribosomal RNA genes as well as genes required for viability and fertility (Gatti and Pimpinelli 1992). Regions necessary for essential chromosomal inheritance functions, including kinetochore formation, sister-chromatid cohesion, disjunction of achiasmate chromosomes, and meiotic pairing of the *X* and *Y* chromosomes, are present in heterochromatin (McKee and Karpen 1990; Hawley *et al.* 1993; Dernburg *et al.* 1996b; Karpen *et al.* 1996; Lee and Orr-Weaver 2001; Sullivan *et al.* 2001). Without an in-depth understanding of heterochromatin structure, a truly complete understanding of genome function and evolution will remain elusive.

The nucleotide sequence of most of the euchromatic

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portion of the *Drosophila melanogaster* genome has been determined (Adams *et al.* 2000), yet progress in understanding the molecular structure and composition of heterochromatin has lagged behind. Approximately one-third of the *D. melanogaster* genome is heterochromatic, yet only a few regions of heterochromatin have been sequenced or mapped molecularly (Devlin *et al.* 1990; Trapitz *et al.* 1992; Hochstenbach *et al.* 1994; Le *et al.* 1995; Losada *et al.* 1997; Sun *et al.* 1997). The preponderance of repetitive sequences in the heterochromatin creates challenges to cloning and sequence analysis, which are difficult to overcome using standard molecular methodologies. The introduction of single-copy molecular-genetic entry points can greatly facilitate structure and sequence analysis of regions rich in repeated DNA. For example, studies of the minichromosome *Dp(1,f)1187* (*Dp1187*) utilized rearrangements between heterochromatin and euchromatin to dissect the structure and inheritance functions of centric heterochromatin (Karpen and Spradling 1990; Le *et al.* 1995; Murphy and Karpen 1995; Sun *et al.* 1997).

Transposition of marked transposable elements provides another method for introducing single-copy entry points into regions of repeated DNA. Indeed, *P*-element transposon insertions were instrumental in assembling a 10-kb sequence of repeated DNA in the *Dp1187* subtelomeric heterochromatin (Karpen and Spradling 1992). The Drosophila *P* element is particularly useful because transposition can be controlled (Spradling and Rubin 1982; Engels 1984; Cooley *et al.* 1989) and modified elements can be constructed *in vitro* and introduced into the genome (Rubin and Spradling 1982). A large collection of euchromatic *P* insertions has been generated and has been invaluable for analysis of euchromatic gene structure and function (Spradling *et al.* 1995, 1999; Rorth *et al.* 1998; Liao *et al.* 2000; Oh *et al.* 2003). Our goal is to generate a large collection of heterochromatic *P* insertions that will serve as molecular-genetic entry points for mapping, sequencing, and functional analysis of Drosophila heterochromatin. This approach requires the development of an effective and robust method for isolating heterochromatic insertions.

P elements have a marked tendency to be recovered in euchromatic rather than in heterochromatic sites (Berg and Spradling 1991). Nevertheless, previous investigations demonstrated that *P*-element insertions can be recovered in heterochromatin by selecting for position-effect variegation (PEV) or by silencing of marker genes contained in the transposon construct. PEV is the clonal inactivation of a euchromatic gene that has been positioned close to or within heterochromatin due to chromosome rearrangement or transposition (reviewed in Karpen 1994; Weiler and Wakimoto 1995; Zhimulev 1998). In Drosophila, reporter genes exhibit suppressed and variegated expression when inserted into centric and telomeric regions but rarely after insertion in euchromatic sites. Heterochromatic insertions have been recovered using silencing of constructs carrying *rosy*$^+$ (*ry*$^+$), *white*$^+$ (*w*$^+$) eye color genes, or *yellow*$^+$ (*y*$^+$) body color and *w*$^+$ genes (Zhang and Spradling 1994; Roseman *et al.* 1995; Wallrath and Elgin 1995; Cryderman *et al.* 1998; Zhang and Stankiewicz 1998). However, these insertions were isolated at a severely reduced rate relative to euchromatic insertions, 2- to 15-fold less than expected on the basis of the physical proportion of heterochromatin in the genome. Furthermore, many of the variegating insertions were recovered in telomeric rather than in centric regions.

In a previous study we showed that screening for variegation of *y* is a very efficient method for recovering centric insertions (Yan *et al.* 2002). Nearly all (97%) of the 73 variegating insertions analyzed in the Yan *et al.* (2002) screen were located in centric heterochromatin. High-resolution fluorescent *in situ* hybridization (FISH) mapping showed that insertions were recovered in 23 of the 61 cytogenetic bands of Drosophila centric heterochromatin. Thus, these results demonstrated proof that high-efficiency recovery of centric heterochromatin *P* elements can be achieved by choosing a marker gene with a strong promoter, to partially ameliorate gene repression (Yan *et al.* 2002). The distribution of insertions within heterochromatin was wide, but nonrandom. For example, insertions in *X* and fourth chromosome heterochromatin, and in much of the third chromosome heterochromatin, were not recovered.

Here we report the results of large-scale screens for *P*-element insertions into Drosophila heterochromatin using *y*$^+$ as a marker gene. Different genetic schemes were used to increase the yield and spectrum of insertion sites. Transposition and selection of variegating insertions were performed under conditions of suppressed and unsuppressed variegation. In addition, transpositions were performed in male and female germlines, and *P* elements were mobilized from both euchromatic and heterochromatic starting positions. All of these schemes produced high frequencies of variegating insertions, especially when the starting site was in heterochromatin. In total, 502 variegating insertions have been produced in these screens; this collection will serve as a key reagent for future studies of heterochromatin structure, sequence, and function.

MATERIALS AND METHODS

Drosophila stocks and culture: Flies were grown on standard cornmeal/molasses/agar media (Ashburner 1990) at 22°. Unless stated otherwise, the mutations and chromosomes used in this study are described in FlyBase (2003). The following standard laboratory stocks were used: (1) *y*^{1}; *ry*^{506}, (2) *y*^{1}; *Sp/CyO,[SUPor-P]*; *ry*^{506}, (3) *y*^{1}; *TMS, P[ry*$^+$*Δ2-3] ry*^{506}*/ry*^{506}, (4) *Y*^{S} *X.Y*^{L}, *In(1)EN, y*^{1}*/Y; ry*^{506}, (5) *Y*^{S}*X.Y*^{L}, *In(1)EN1, y*^{1}*/0; TMS, P[ry*$^+$*Δ2-3] ry*^{506}*/ry*^{506}, (6) *Y*^{S}*X.Y*^{L}, *In(1)EN1, y*^{1}*/Y; TMS, P[ry*$^+$*Δ2-3] ry*^{506}*/ry*^{506}, and (7) *C(1)RM, y*^{1} *v*^{1}*/Y; ry*^{506}. With the exception of dominantly marked or rearranged chromosomes, all these stocks shared the same *y*^{1}; *ry*^{506} genetic back-

ground. The *$Y^S X.Y^L$, In(1)EN1, y* attached *X-Y* chromosome is further designated as *X^Y* and is used to introduce an extra *Y* chromosome for PEV suppression. *C(1)RM, y^1 v^1* is composed of two *X* chromosomes attached to a single centromere. The strain containing the *SUPor-P* (**sup**press**or-P**) element at polytene chromosome position 60F on the *CyO* balancer chromosome is described by Roseman *et al.* (1995). The *SUPor-P* element carries two reporter genes, y^+ and w^+, as well as two *suppressor of hairy wing* [*su(Hw)*] binding regions (chromatin insulator elements from the *gypsy* transposon) flanking the w^+ gene (Roseman *et al.* 1995). We consider the 60F insertion to be located in euchromatin; the insertion is subterminal, and the *yellow* and *white* marker genes are fully expressed and exhibit none of the variegation observed for telomeric insertions. In addition, we used six stocks carrying variegating *Y* chromosome *SUPor-P* insertions, as described in Yan *et al.* (2002): B783.2 (insertion in h17–18), B840.1 (h10), J632.2 (h10–13), B296 (h16), K13.1 (h11–13), and C151(h3). All *P* elements were mobilized using the *P[$ry^+\Delta 2$-3]*(99B) transposase present on the *TMS* balancer chromosome (Robertson and Engels 1989).

Definition of the *y* variegation phenotype: In a previous study we demonstrated that insertions could display two types of aberrant expression of *y* (Yan *et al.* 2002). The first type, referred to as "*y* misexpression" insertions, displayed either a general lightening of the pigmentation in the wings and/or the abdomen compared to wild-type or specific patterns of pigmentation (for example, *y* wings/wild-type abdomen or the opposite, gradients in pigmentation level, patches of lighter, but not *y* cuticle). The second type, the "*y* variegators," exhibited predominantly *y* abdomens with y^+ spots or predominantly y^+ abdomens with patches of *y* pigmentation. In this study we isolated only "*y* variegating" insertions with clear all-or-none mosaic *y* expression, since previous FISH analysis demonstrated that "*y* misexpression" insertions all localized to euchromatin or telomeres (Yan *et al.* 2002). Note that nonvariegating, primarily euchromatic insertions were saved and incorporated into the Drosophila Gene Disruption Project ("KG" lines, http://flypush.imgen.bcm.tmc.edu/pscreen/).

Genetic screens to isolate heterochromatic insertions: Nine different mating schemes were used to isolate *yellow*-variegating insertions (Figure 1). For the sake of simplicity, the experiments involving particular genetic schemes are referred to hereafter as "scheme" with the corresponding number. For all experiments, F_1 progeny used to activate transposition were produced by crosses *en masse* in bottles. The mobilization-generating crosses were performed in vials as a precaution against recovering multiple lines from the same insertion event. Unless stated otherwise, F_1 transposition generations involved crossing four virgin females with three to four males. Such experimental conditions are optimal for producing a high yield of insertions, without generating too many events in one vial (Dobie *et al.* 2001).

In genetic schemes 1–6, the *SUPor-P* element inserted on the *CyO* chromosome at cytological location 60F was used as a starting point for transposition. In all these schemes new mobilizations were identified in the F_2 generation as straight-winged flies (Cy^+) with pigmented wings and/or abdomens. Variegating flies, presumably arising as a result of simultaneous excision of the element and insertion into heterochromatin, could also be detected among Cy progeny. Therefore, flies with *y* variegation were selected regardless of the wing phenotype. However, variegators recovered from Cy flies were excluded from all calculations of transposition frequency, because corresponding nonvariegating transpositions could not be distinguished from the flies bearing the original insertion in the *CyO* chromosome.

Summaries of the genotypes used in the mobilization and selection generations are shown in Figure 1. The rationale and crosses used for each scheme are detailed as follows.

Schemes 1–6 utilized the *SUPor-P* in 60F as the starting point for transposition:

Scheme 1. This is the same scheme used in the original (Yan *et al.* 2002) study, involving mobilization in males, and no extra *Y* chromosome in either the transposition or the scoring generations. *SUPor-P* was activated by a transposase source (Δ2-3) in the male germlines. These males were produced by crossing *y^1; Sp/CyO, P{SUPor-P}*; *ry^{506}* females either to *y^1; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* males or, in some experiments, to *$Y^S X.Y^L$, In(1)EN, y^1/Y; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* males. Males carrying both the *SUPor-P* element and *TMS, Δ2-3* chromosomes were mated to *y^1*; *ry^{506}* females and progeny were screened for new insertions.

Scheme 2. Transpositions from 60F were induced in regular *X/X* females to compare frequencies of recovery from females *vs.* males. Virgin *y^1; +/CyO, P{SUPor-P}; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* F_1 females were crossed to *y^1*; *ry^{506}* males. F_1 females were generated by crossing *y^1; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* females to *y^1; Sp/CyO, P{SUPor-P}*; *ry^{506}/ry^{506}* males.

Scheme 3. Mobilization occurred in males with no extra *Y*, and new mobilization events were screened in flies carrying an additional *Y* chromosome. *y^1; +/CyO, P{SUPor-P}; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* F_1 males were crossed to *$Y^S X.Y^L$, In(1)EN, y^1*; *ry^{506}* females.

Scheme 4. Mobilization occurred in normal genotype females, and new events were scored in females with additional heterochromatin and in males with a normal sex-chromosome constitution. Virgin F_1 females of genotype *y^1; +/CyO, P{SUPor-P}; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* were crossed to *$Y^S X.Y^L$, In(1)EN, y^1/Y*; *ry^{506}* males.

Scheme 5. Females of the genotype *$Y^S X.Y^L$, In(1)EN, y^1; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* were crossed to *y^1; Sp/CyO, P{SUPor-P}* males to generate males bearing *SUPor-P, Δ2-3*, and an additional *Y* chromosome. These males were crossed to *$Y^S X.Y^L$, In(1)EN, y^1*; *ry^{506}* to select for mobilization events in the presence of an additional *Y* chromosome.

Scheme 6. To produce mobilization-generating females with an additional *Y* chromosome, *y^1; Sp/CyO, P{SUPor-P}* females were crossed to *$Y^S X.Y^L$, In(1)EN1, y^1/0; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* males. Males generated in these crosses were sterile due to lack of a *Y* chromosome, which facilitated virgin selection. *$Y^S X.Y^L$, In(1)EN, y^1/y^1; +/CyO, P{SUPor-P}; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* females were mated to *$Y^S X.Y^L$, In(1)EN, y^1/Y*; *ry^{506}* males to select for new mobilizations in the presence of an extra *Y* chromosome.

Schemes 7–9 utilized heterochromatic insertions as starting points for remobilization:

Scheme 7. *$Y^S X.Y^L$, In(1)EN, y^1/Y, P{SUPor-P}; TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* mobilization males with an extra *Y* chromosome were obtained by crossing *$Y^S X.Y^L$, In(1)EN, y^1*; *TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* females to *y^1/Y, P{SUPor-P}*; *ry^{506}* males. Six lines carrying *Y* chromosome variegating *SUPor-P* insertions in different locations were used in these experiments. *X^Y/Y* males carrying variegating *Y* chromosome insertions and transposase were mated to *C(1)RM, y^1 v^1/Y*; *ry^{506}* females. Half of the progeny died as result of aneuploidy; therefore, five to seven virgin females instead of four were used to increase the yield of progeny. New insertions were identified as y^+-expressing males in the presence of an extra *Y* chromosome.

Scheme 8. In this scheme *Y* chromosome *SUPor-P* insertions were mobilized in males with a regular sex-chromosome constitution. F_1 males of genotype *y^1/Y, P{SUPor-P}*; *TMS, P[$ry^+\Delta 2$-3] ry^{506}/ry^{506}* were selected after crossing *y^1*; *TMS,*

P[ry^{+}Δ2-3] ry^{506}/ry^{506} females to *y^{1}/Y, P{SUPor-P}; ry^{506}* males bearing the K13.1 or B783.2 *Y* chromosome insertions. Mobilization events were recovered as pigmented males produced in the crosses of *C(1)RM, y^{1} v^{1}/Y; ry^{506}* females to *y^{1}/Y, P{SUPor-P}; TMS, P[ry^{+}Δ2-3] ry^{506}/ry^{506}* males. Crosses were performed using the same conditions as described for scheme 7.

Scheme 9. Two *y* variegating *SUPor-P* insertions in the heterochromatin of the *CyO* chromosome, for simplicity referred to as *CyO, y^{var}*, were used as the source for mobilization. Three or four F_1 *Y^{S}X.Y^{L}, In(1)EN, y^{1}/Y; +/CyO, y^{var} ; TMS, P[ry^{+}Δ2-3] ry^{506}/ry^{506}* males were mated to four *Y^{S}X.Y^{L}, In(1)EN1, y^{1}; ry^{506}* virgin females. New transpositions were identified in the F_2 as straight-winged pigmented flies in the presence of an extra *Y* chromosome.

Stock establishment and determination of genetic linkage: Since variegating insertions are relatively rare events, we selected all variegating flies, males and females, with and without the *TMS* balancer (*Sb* and *Sb*$^{+}$). Stocks were established by backcrossing to either *y^{1}; ry^{506}* or *Y^{S}X.Y^{L}, In(1)EN, y^{1}; ry^{506}*, depending on the mating scheme. If insertions were recovered as females, male F_3 progeny from variegating females were used to establish the stocks. For variegators recovered as females in scheme 3, backcrosses to *Y^{S}X.Y^{L}, In(1)EN, y^{1}; ry^{506}* females were repeated twice to ensure uniform sex-chromosome constitution of the stocks. Variegating insertions into the *TMS* balancer were discarded. For non-*TMS* insertions recovered as *Sb* flies, the *TMS* chromosome was removed by crosses with either *y^{1}; ry^{506}* or *X^Y, y^{1}; ry^{506}*. To avoid remobilization or rearrangement of insertions, 5–10 substocks were established from individual *Sb*$^{+}$ variegating males derived from these crosses. When the level of variegation among substocks was identical, one substock was kept as representing the original insertion. Occasionally we observed the appearance of flies with a different variegation phenotype from the majority or with a different genetic linkage of the insertion; such flies were used to make independent stocks of likely secondary transpositions. These stocks are not included in Tables 1–3, but are reported in the text and are included in the total number of insertions generated. All stocks carrying variegating insertions were maintained by crosses of variegating flies *inter se*. Genetic linkage of insertions with *Y, X, X^Y*, or autosomes was determined by analysis of marker inheritance.

Statistical analysis: Considering that the yield of flies and ratios of flies of different genotypes varied among the schemes, we calculated the frequency of transpositions relative to the total number of flies screened (Figure 1, Table 3). Since *P*-element mobilization often occurs premeiotically (Daniels and Chovnick 1993), multiple flies with new transpositions recovered from the same vial were counted as a single transposition event. Thus, the transposition rate may be underestimated, since three to four males and female parents were used in transposition-generation crosses. This should have very little impact on the frequency of variegating insertions, because the recovery of variegators per vial was low. Chi-square tests for independence were used to estimate the statistical significance of differences in the frequency of transpositions and the proportion of variegating insertions.

FISH localization: Insertions were localized with respect to the 61 heterochromatic bands in mitotic chromosomes from larval neuroblasts, using *SUPor-P* as the probe. Methods used for FISH and qualitative and quantitative assignments to bands are described in Yan *et al.* (2002).

RESULTS

Design of the screens: In a previous study we determined that screening for *y* variegation among insertions of *SUPor-P* is a very efficient method for recovering centric insertions (Yan *et al.* 2002). In this study we conducted large-scale screens for *y*-variegating *SUPor-P* insertions to produce multiple, unique entry points into Drosophila centric heterochromatin. The relatively low frequency of heterochromatic insertions compared to insertions into euchromatin observed in previous screens could be caused by a reduced probability of insertion in heterochromatin due to the condensed nature of the chromatin or the absence of insertion sites preferred by *P* elements. Alternatively, the recovery of heterochromatic insertions could be reduced due to complete silencing of the marker genes. Nine different genetic schemes (see materials and methods and Figure 1) were carried out to determine if modification of genotypes during mobilization and selection of insertions would alter the yield of variegators and the spectrum of insertion sites. Some schemes used a strong suppressor of PEV (an extra *Y* chromosome) in the scoring generation to recover insertions that would otherwise be phenotypically silenced (Zhang and Spradling 1994). Other schemes included an extra *Y* in the mobilization generation to test the hypothesis that "opening" the chromatin would cause heterochromatin to be a better target for insertion. The starting site could also affect the integration or recovery of heterochromatic *P*'s. For example, heterochromatic regions associate with each other in the nucleus (Dernburg *et al.* 1996a), and transposition from a heterochromatic position may increase the probability of integration into heterochromatin. Therefore, we compared mobilization of *SUPor-P* from a euchromatic site (60F) and heterochromatic sites (*Y* or second chromosome). Finally, mobilization was carried out in males and females to determine if there were gender-specific differences in the mobilization or recovery of variegating insertions. The specific crosses used are described in materials and methods and Figure 1.

Mobilization of *SUPor-P* from a euchromatic position (*CyO* chromosome, position 60F): *Mobilization and selection in males and females with regular sex-chromosome constitution (schemes 1 and 2):* Scheme 1 involved mobilization in regular *X/Y* males and selection of variegating insertions among flies with a normal sex-chromosome constitution. Fourteen percent of the insertions recovered in scheme 1 displayed *y* variegation. This proportion is nearly fourfold higher than that observed in the pilot study (3.1%; Yan *et al.* 2002), most likely due to experience gained in the pilot screen that facilitated the identification of variegators. For individual established lines, variegation was usually much stronger in females *vs.* males; in some cases there appeared to be no *y*$^{+}$ expression in females, whereas their male siblings displayed visible *y* variegation. This observation can account for the large difference in the recovery of variegating females (14% of variegators) *vs.* variegating males (86%; $P < 0.01$, expect a 1:1 ratio, Table 1). Sex-specific differences in *y* expression could also explain why we failed

SCHEME	SOURCE	TRANSPOSITION	SELECTION ♀	SELECTION ♂	# SCREENED	HOPS #	HOPS %	VARIEGATORS #	VARIEGATORS %
1	60F	X/Y ♂	X/X	X/Y	186517	618	0.57	74	12
2	60F	X/X ♀	X/X	X/Y	29249	109	0.66	4	4
3	60F	X/Y ♂	X/X^Y	X^Y/Y	201296	778	0.61	115	15
4	60F	X/X ♀	X/X^Y	X/Y	39014	126	0.50	3	2
5	60F	X^Y/Y ♂	X^Y/X^Y	X^Y/Y	200898	985	0.74	176	18
6	60F	X^Y/X ♀	X/X, X/X^Y	X/Y, X^Y/Y	15340	682	0.68	35	5
7	Y het	X^Y/Y ♂		X^Y/Y	37149	172	0.46	64	37
8	Y het	X/Y ♂		X/Y	49275	111	0.22	28	25
9	2 het	X^Y/Y ♂	X^Y/X^Y	X^Y/Y	56996	191	0.34	50	26
				TOTALS	954734	3772	0.40	549	16

FIGURE 1.—Description of the screens and results. Genotypes of animals used during the transposition and selection stages are shown for the different schemes. The results for each scheme are reported in the right-hand columns, including the total number of flies screened, the total number of transpositions (hops), the percentage of animals with hops, the total number of variegating insertions, and the percentage of all insertions that were variegators. See MATERIALS AND METHODS for exact genotypes and screening methods. Note that the data presented here do not include insertions recovered in phenotypically Cy animals, because the corresponding fully *y*+ new insertions in Cy animals could not be identified. These lines are included in the totals reported in Tables 1–3.

to isolate *X* chromosome centric insertions in the YAN *et al.* (2002) pilot study and why only 2 of the 90 variegating insertions identified in scheme 1 were *X* linked (2%, Table 1). Any insertions into the *X* in scheme 1 would have to be recovered in female progeny, where the yellow phenotype is less visible. Scheme 1 resulted in the establishment of 67 stocks with variegating insertions (Table 1).

We mobilized the *P* element in females (scheme 2) to assess potential sex-specific differences in the mobilization of *P* elements into heterochromatic sites. We were also interested in determining if more *X* centric insertions would be selected when mobilization events to the *X* chromosome were recovered in males. The overall insertion frequencies were similar in schemes 1 and 2 (0.57 *vs.* 0.66%, respectively, Figure 1). However, a significantly lower percentage of variegating insertions (4%) was observed when *SUPor-P* was mobilized in females compared to its mobilization in males (12%; $P < 0.01$; Figure 1). Interestingly, one of four variegating insertions recovered in scheme 2 was *X* linked (*vs.* only 2% from *X/Y* males, Table 1); although the numbers are low, this result suggests that mobilization in females may enhance recovery of insertions in *X* centric heterochromatin. We conclude that recovery of variegators is significantly higher when mobilization occurs in males *vs.* females with normal sex-chromosome constitutions.

Mobilization in males and females with regular sex-chromosome constitution and selection in the presence of an additional Y chromosome (schemes 3 and 4): The *Y* chromosome acts as a potent suppressor of PEV *in trans*, presumably by diluting heterochromatic proteins and increasing accessibility of transcription factors (DIMITRI and PISANO 1989). Does selection of insertions in the presence of an additional *Y* chromosome affect the recovery of variegating insertions? Selection under PEV-suppressed conditions of variegating insertions produced by mobilization of the 60F *SUPor-P* element in males with a regular sex-chromosome constitution (scheme 3) resulted in slightly higher recovery of variegators in comparison to scheme 1 (15 *vs.* 12%, respectively, Figure 1), but the difference is not statistically significant ($P > 0.1$). In total, 99 stocks with variegating insertions were established from scheme 3 (Table 1). As in scheme 1, a significantly higher proportion of variegators were recovered in males *vs.* females (74 *vs.* 26%, Table 1). Interestingly, we observed an approximately twofold increase in the recovery of variegating females in comparison to scheme 1 (26 *vs.* 12%, Table 1); however, a larger sample would be required to prove that this difference was significant.

Recovery of variegating insertions was very low (2% of total insertions) when mobilization occurred in normal sex-chromosome constitution females, and females were scored in the presence of an extra *Y* (scheme 4); only two variegating males were recovered. This result confirmed that mobilization of the 60F *SUPor-P* element in females produces a significantly lower proportion of variegating insertions than mobilization in males produces (see above).

TABLE 1

Genetic characterization of variegating insertions

Scheme	No. of variegating insertions	No. of established stocks[a]	Recovery sex (% of total)		Location (% of total)						
									Autosomes		
			M	F	*X*	*Y*	*X^Y*	*X* or *X^Y*[b]	*CyO*	*TMS*	Other
1	90	67	86	14	2	10	NA	NA	8	18	62
2	4	4	75	25	25	NA	NA	NA	0	0	75
3	139	99	74	26	0	25	NA	NA	12	13	50
4	3	3	100	0	0	NA	NA	NA	0	33	66
5	203	175	72	28	NA	12	8	NA	8	8	63
6	41	38	66	34	NA	NA	NA	12	2	12	73
7	55	50	100	NA	NA	NA	44	NA	NA	9	47
8	21	17	100	NA	10	NA	NA	NA	NA	29	62
9	57	49	70	30	NA	26	2	NA	NA	12	59
Total	**613**	**502**	**77**	**23**	**1**	**13**	**6**	**1**	**6**	**12**	**59**

All genetically characterized insertions are shown, including those that were not included in established stocks because they were subsequently lost or were not kept because they were inserted on the *TMS* or *CyO* balancers. Also included are variegating insertions selected as Cy flies, which were omitted from Figure 1 (see its legend); most of these were new insertions in the *CyO* chromosome, which were discarded, but some were demonstrated to be on other chromosomes during subsequent outcrosses and thus were included in the established stocks.

[a] Established stocks do not include variegating insertions on the *CyO* and *TMS* balancer chromosomes or any that were lost or sterile. Although new insertions on *CyO* were kept, we have not included them in the totals because they are likely to be less useful in future analysis.

[b] Insertions are linked with either the *X* or the *X^Y* chromosomes.

Mobilization of P elements in the presence of an additional Y chromosome (schemes 5 and 6): We were interested in determining if the presence of an extra *Y* in the mobilization generation would increase the probability of insertion into heterochromatin and thus the recovery of variegating insertions. Scheme 5 involved mobilization in males with an extra *Y* chromosome and selection under conditions of PEV suppression (Figure 1). The proportion of variegating males of the same *X^Y/Y* genotype recovered from schemes 3 and 5 did not differ significantly (22 *vs.* 24%, Table 2, $P > 0.05$). The proportion of variegating flies among *X^Y/X^Y* females also did not differ significantly from the proportion of variegators recovered among *X^Y/X* females (10 *vs.* 7%, Table 2).

However, the frequency of variegating insertions was increased significantly when an additional *Y* chromosome was present during both mobilization and recovery. The proportion of variegating insertions among *X^Y/Y* males and *X^Y/X^Y* females recovered in scheme 5 was significantly higher than the proportions of variegating insertions among flies with regular sex-chromosome constitution selected in scheme 1 (24 *vs.* 19% for males, $P < 0.05$, and 10 *vs.* 4% for females, $P < 0.01$, respectively, Table 2). Interestingly, the frequency of all transposition events (variegating and nonvariegating insertions) was significantly higher when transposition occurred in *X^Y/Y* males (0.74% in scheme 5 *vs.* 0.57% in scheme 1 and 0.61% in scheme 3, $P < 0.01$, Figure 1). Scheme 5 produced the highest yield of variegating insertions: 175 stocks with variegating insertions were established, including 16 insertions on the *X^Y* chromosome (Table 1; 8% of variegating insertions).

By contrast, mobilization of *SUPor-P* in females with extra heterochromatin and selection under conditions where PEV is suppressed in half of the progeny (scheme 6, Figure 1) did not significantly increase recovery of variegating insertions (5%) in comparison to schemes 2 (4%) and 4 (2%; $P > 0.05$, Figure 1). Scheme 6 females produced a lower frequency of variegating insertions than that observed in any scheme where *SUPor-P* transposed in males. Nevertheless, 38 variegating stocks were established from scheme 6.

Mobilization of *SUPor-P*'s located in different regions of heterochromatin: *Mobilization of Y chromosome SUPor-P insertions (schemes 7 and 8): P* elements mobilize preferentially to nearby regions of the homologous chromosome (Tower and Kurapati 1994). We reasoned that this might result from a compartmentalization within the nucleus; previous investigators showed that different regions of heterochromatin associate in the nucleus (Dernburg *et al.* 1996a). Therefore, we determined whether mobilization from heterochromatic locations might increase the proportion of heterochromatic insertions among total transposition events.

Transpositions of six *Y* insertions (see materials and methods and Yan *et al.* 2002) were generated in males carrying an extra *Y* chromosome (scheme 7, Figure 1, and materials and methods). We observed up to a sixfold difference between starting *Y* insertions in the

TABLE 2

Effects of an extra *Y* chromosome during mobilization and recovery

Scheme	Chromosome constitution at mobilization	Chromosome constitution at recovery:	Variegating insertions [total no. of insertions (%)[a]]						
			X/Y	*X^Y/Y*	*X^Y/Y* or *X/Y*	*X/X*	*X^Y/X*	*X^Y/X^Y*	*X^Y/X^Y* or *X^Y/X*
1	*X/Y; CyO, y*$^+$ 60F		19			4			
3	*X/Y; CyO, y*$^+$ 60F			22			7		
2	*X/X; CyO, y*$^+$ 60F		6			2			
4	*X/X; CyO, y*$^+$ 60F		4				0		
5	*X^Y/Y; CyO, y*$^+$ 60F			24				10	
6	*X^Y/X; CyO, y*$^+$ 60F				6				4
7	*X^Y/Y, y*^{var}			37					
8	*X/Y, y*^{var}		25						
9	*X^Y/Y; CyO, y*^{var}			33				18	

[a] The percentage of variegating insertions recovered in each genotype is normalized to the total number of insertions recovered in the relevant sex. For example, for the *X/Y* column, the number of variegating insertions recovered in *X/Y* males was divided by the total number of insertions (variegating and nonvariegating) recovered in all males and multiplied by 100 to produce the percentage. Blank spaces indicate that the genotype was not present in the scheme.

overall frequency of transpositions (range 0.11–0.69%; average 0.46%; Table 3). Some *Y* insertions, especially J632.2, showed significantly reduced rates of transposition compared to the 60F starting site (Figure 1 and Table 3). In contrast, K13.1 generated a similar frequency of transpositions to that observed in scheme 5. Most importantly, the overall proportion of variegating insertions was twofold higher for mobilization of *SUPor-P* from starting sites in the *Y* chromosome (scheme 7, 37%, Figure 1 and Table 3) compared to mobilization from 60F in males with the same sex-chromosome constitution (scheme 5, 18%, Figure 1; $P < 0.01$). The proportion of variegating insertions differed among the *Y* chromosome starting insertions (range 29–54%, Table 3), but all six produced a significantly higher proportion of variegators than that observed for the 60F starting site in scheme 5 or in any other scheme (Figure 1).

Interestingly, the six *Y* insertions also produced different distributions of new insertion sites. For example, 10 of 14 insertions recovered from line B840.1 were located in the *X^Y* chromosome (2 insertions were recovered in sterile males and therefore were not localized), while only 3 *X^Y* insertions were recovered in 20 genetically characterized insertions from line K13.1 (Table 3, $P < 0.01$). Thus, the heterochromatic starting site appears to affect both overall transposition frequency and new insertion-site preference.

Overall, we established 64 variegating lines from scheme 7; 24 (44%) were insertions in the *X^Y* chromosome. In addition to insertions recovered in this scheme as *y*$^+$-expressing males, we recovered 17 lines from females with increased or decreased variegation, compared to the original insertion. These changes were heritable and *Y* linked (Table 3). Such changes in the level of variegation might result from remobilization of the insertions into the same chromosome, from local duplications of the *P*, or from changes (*e.g.*, deletions) in the vicinity of the insertion. Further analysis is necessary to determine if these lines contain useful new heterochromatic insertions; thus, these lines are not included in our estimates of new centric insertions produced by these schemes.

We also mobilized two different *Y* chromosome *SUPor-P* insertions in males with a regular sex-chromosome constitution (scheme 8; see Figure 1 and MATERIALS AND METHODS). For both insertions (K13.1 and B783.2), the overall frequency of transpositions was nearly twofold lower than that in scheme 7, where mobilization occurred in the presence of an extra *Y* chromosome (Table 3, $P < 0.01$). The relative difference in transposition rate between K13.1 and B783.2 remained the same in the presence and absence of an additional *Y* chromosome. Interestingly, the proportion of variegating insertions recovered in *X/Y* males was significantly higher for the B783.2 line than that observed for the euchromatic 60F starting site (41%, B783.2, scheme 8 *vs.* 19%, 60F, scheme 1, $P < 0.01$, Tables 2 and 3), but did not differ for K13.1 (17 *vs.* 19%). K13.1 produced a significantly higher proportion of variegating insertions in the presence of an additional *Y* chromosome during mobilization (31%, scheme 7 *vs.* 17%, scheme 8; $P < 0.05$, Table 3). Therefore, the frequency of transposition from heterochromatic starting sites appears to be determined by the location of the insertion and is increased significantly when an extra *Y* chromosome is present during mobilization, regardless of the initial starting site. Seventeen variegating lines were established from scheme 8 (9 from B783.2 and 12 from K13.1).

Remobilization of variegating insertions in the CyO chromosome: To test whether increased recovery of variegators

TABLE 3

Mobilization of *SUPor-P* from heterochromatic positions

Starting insertion				Hops		Variegating insertions		Chromosome location[d]			
Line	Location	Scheme	No. of flies[a]	No.	%[b]	No.	%[c]	Autosomes	*X^Y*	*CyO*[e]	*Y*[e]
B783.2	*Y* h17-18	7	4,995	13	0.26	7	54	2	4	NA	4
B783.2		8	26,862	39	0.15	16	41	9	NA	NA	1
K13.1	*Y* h11-13	7	10,295	71	0.69	22	31	17	3	NA	3
K13.1		8	22,413	72	0.35	12	17	12	NA	NA	0
B840.1	*Y* h10	7	6,246	41	0.66	16	39	4	10	NA	0
J632.2	*Y* h10-13	7	5,928	7	0.11	2	29	1	1	NA	1
B296	*Y* h16	7	3,740	15	0.40	6	40	3	1	NA	1
C151	*Y* h3	7	5,945	25	0.42	11	44	4	5	NA	13
Subtotal		7	37,149	172	0.46	64	37	31	24	NA	22
		8	49,275	111	0.22	28	25	21	0	NA	1
Total			**86,424**	**283**	**0.33**	**92**	**33**	**52**	**24**	**NA**	**23**
IIIA	*2* het	9	16,232	83	0.51	20	24	17	0	14	5
IIIB	*2* het	9	40,764	108	0.27	30	28	18	1	86	11
Subtotal			56,996	191	0.34	50	26	35	1	100	16
Total			**143,420**	**474**	**0.33**	**142**	**30**	**87**	**18**	**100**	**39**

[a] Total number of males (schemes 7 and 8) and Cy^+ flies (scheme 9) screened (see MATERIALS AND METHODS).
[b] Percentage of flies that contained new identifiable insertions.
[c] Percentage of all hops that variegated.
[d] Chromosome location for some new insertions could not be determined because selected variegating flies were sterile.
[e] For the cases where the starting insertion was on the *Y* (schemes 7 and 8) or on the *CyO* chromosome (scheme 9), these new *Y* and *CyO* insertions were selected due to changes in the level of variegation.

is a general property of heterochromatic starting insertions, we remobilized two variegating insertions in the *CyO* chromosome. Insertions IIIA and IIIB were recovered in experiments using genetic schemes 1 and 5, respectively, presumably resulting from excision of *SUPor-P* from 60F and reinsertion in the *CyO* heterochromatin. Transposition and recovery of variegators were performed under conditions of suppressed PEV (scheme 9; Figure 1 and MATERIALS AND METHODS). The two insertions showed an approximately twofold difference in the transposition rate, but the proportion of variegating insertions was similar (24 and 28%, Table 3). As for the *Y* chromosome insertions, we observed a significant increase (26%) in the overall proportion of variegating insertions compared to mobilization of the euchromatic 60F insertion in the same background (26 *vs.* 18%, scheme 5; $P < 0.01$, Figure 1, Table 3). Interestingly, only 2% of variegating insertions were located in the *X^Y* compound chromosome, which comprises ~35% of all the heterochromatin in *X^Y/Y* males. In comparison, 44% of the variegating insertions generated from *Y* starting sites (scheme 7) were located in the *X^Y*, and 8% were generated from the 60F euchromatic starting site in the *CyO* chromosome (scheme 5, $P < 0.05$; see Tables 1 and 3). However, the proportion of *Y* chromosome insertions was significantly increased compared to scheme 5 (15% of all insertions in scheme 9 *vs.* 5% in scheme 5, $P < 0.01$). In total, 49 stocks carrying variegating insertion chromosomes were established from scheme 9.

We also frequently recovered Cy flies with a consistent and heritable change in the level of variegation in comparison to the original insertions. Progeny of 107 independently recovered *CyO* flies with increased variegation were studied. Most of these insertions were linked to the *CyO* chromosome, but 7 represented insertions in another chromosome. We propose that *CyO*-linked events represent simultaneous excisions and intrachromosomal transpositions to sites of stronger repression or rearrangements of the sequences surrounding the original insertion. The frequency of these events differed between the two insertions and reached 1/198 *CyO* chromosomes for insertion IIIB.

We conclude that mobilization from eight different heterochromatic insertions leads to a significantly elevated recovery of variegating insertions. It is remarkable that the proportion of variegators relative to all mobilization events is close to or higher than the proportion of the genome that is considered heterochromatic (see DISCUSSION).

TABLE 4

Chromosomal distribution of FISH-localized insertions

	X		*Y*		2		3		4			
Scheme	Obs[b]	Exp[c]	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	*N*[d]	%[e]
P + 1[a]	0	19	22	40	44	18	34	17	0	6	77	54
3	0	19	50	40	17	18	17	17	17	6	12	10
5	0	0	12	49	43	22	45	21	0	8	60	34
6	0	32	0	0	79	30	21	28	0	10	14	40
7	0	0	0	0	50	61	50	29	0	10	6	9
8	33	24	0	0	67	46	0	22	0	8	6	21
9	0	0	46	49	27	22	18	21	9	8	22	44
Total	**1**	**14**	**20**	**26**	**44**	**31**	**33**	**22**	**2**	**8**	**197**	**33**

[a] The results of scheme 1 and the pilot screen (Yan *et al.* 2002) were combined to summarize the results for all KV lines that have been localized.

[b] Percentage of variegating insertions recovered that localized to the heterochromatin in each chromosome. All data reported do not include seven insertions that localized to chromosomes that should not have been recoverable targets in schemes 5 (*X*) and 6–8 (*Y*). It is likely that these insertions were recovered due to meiotic nondisjunction or recombination.

[c] Percentage of insertions expected on the basis of the amount of heterochromatin in each chromosome (Hoskins *et al.* 2002) and the number of each chromosome that could be recovered with an insertion in each scheme.

[d] Total number of insertions localized.

[e] Percentage of variegating insertions recovered in each scheme that have been localized.

FISH localization of variegating insertions: We determined the locations of a subset of the variegating insertions with respect to individual chromosomes and the cytogenetic banding maps using the FISH protocol described in Yan *et al.* (2002). In the pilot screen, we observed that 71 of 73 variegating insertions (97%) were located in the centric heterochromatin and that the remaining 2 insertions were telomeric (Yan *et al.* 2002). We have localized 131 variegating insertions generated by the schemes reported here: 128 were centric (98%), 1 was telomeric, and 2 were in the euchromatic arms.

The distributions of insertions with respect to individual chromosomes and cytogenetic bands provide insights into the effects of the different starting sites and the mobilization and scoring genotypes used in the different schemes. Table 4 compares the observed chromosomal distributions of FISH-localized heterochromatic insertions to the distributions that would be expected if insertions were recovered proportional to the amount of heterochromatin in each target chromosome. In the pilot scheme 1 and scheme 5, second and third chromosome insertions were overrepresented, whereas *Y* and fourth chromosome insertions were underrepresented. In comparison, scheme 9 produced a distribution that was proportional to the total amount of heterochromatin in each chromosome. In addition, our first *X* and fourth chromosome *SUPor-P* insertions were recovered from schemes 8 and 3 and 9, respectively.

We conclude that mobilization from a heterochromatic location, perhaps in combination with the presence of an extra *Y* chromosome in the scoring generation, results in a more uniform gross distribution of insertions in the heterochromatin, in addition to an increased recovery (see DISCUSSION). The number of insertions localized for screens 3, 7, and 8 was too small to make definitive conclusions, but in general was consistent with this hypothesis, as were the genetic mapping results (Tables 1 and 2).

The analysis of insertion sites relative to the heterochromatic bands demonstrates that schemes 2–9 produced a distribution that was significantly broader than that observed for the pilot screen (Yan *et al.* 2002) and scheme 1 (Figure 2). The distribution of insertions from the pilot screen included regions that contained many insertions (*e.g.*, 47–48) and regions with no or very few insertions (*e.g.*, 53–56, *X* and fourth chromosomes). Many of the previous gaps in coverage now contain insertions such that 48 of 61 bands have at least one insertion, compared to 23 of 61 in the pilot screen. In the new schemes, regions 47 and 48 continued to be a hot spot, but insertions were recovered in 53–56, and a new concentration of insertions near the centromere of chromosome 2 (38–41) was observed, which was not present in the pilot screen results. Scheme 5, in which a euchromatic insertion was mobilized and variegators were selected in the presence of an extra *Y*, produced a more even distribution across the second and third chromosome heterochromatic bands, in comparison to the pilot scheme 1 (Figure 2).

DISCUSSION

Here we describe the results of different genetic schemes designed to examine the effects of genetic background

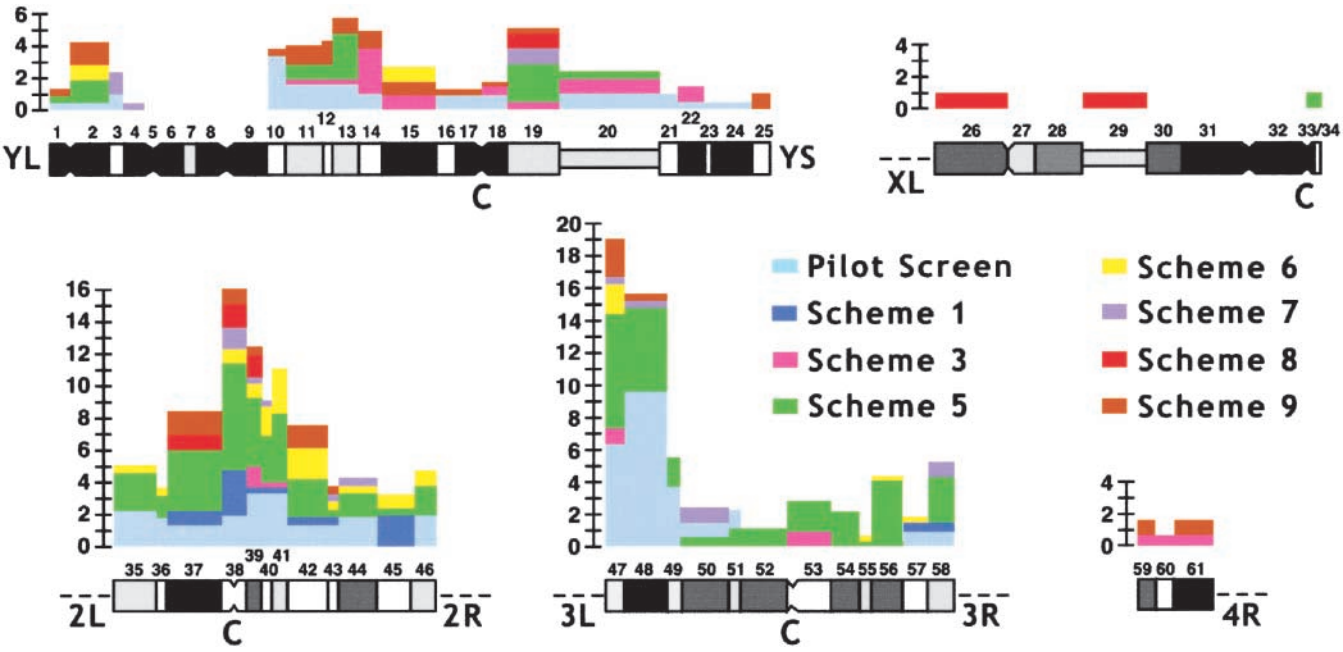

FIGURE 2.—Distribution of variegating *P* insertions in centric heterochromatin. The FISH localization results for schemes 1–9 are presented in combination with the results from the pilot screen (including the seven *X* and *Y* insertions excluded from Table 4; YAN *et al.* 2002). Blocks represent the 61 cytogenetic bands of heterochromatin, h1–h61 (GATTI *et al.* 1994). Bars above each chromosome represent the number of *P* insertions localized to that region (*x*-axis). C, centromere. Some *P* insertions could be localized to only two to four adjacent regions, depending on the resolution of the FISH signal and the 4′,6-diamidino-2-phenylindole banding pattern. 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 two bands, each band was assigned a value of 0.5.

and gender on the recovery of *y*-variegating insertions. In summary, from schemes 1–9 we have established 502 *y*-variegating insertion lines. In addition, we recovered 41 and 75 variegating insertions on the *CyO* and *TMS* balancer chromosomes, respectively, which are not included in our totals or in established stocks because these chromosomes are highly rearranged and would not be as useful in future analysis of the structure and function of centric heterochromatin. We were able to substantially increase the yield of variegating insertions in schemes that utilized different genetic backgrounds. Most notably, the proportion of total insertions that variegated was 17–54% when *SUPor-P* was mobilized from heterochromatic locations (Table 3).

Results of FISH localization to mitotic chromosomes for 131 lines generated in schemes 1–9 have shown that 98% of them are centric, which is nearly identical to the results of our previous pilot screen (YAN *et al.* 2002). A total of 204 variegating insertions have been localized to date, of which 199 are centric (97.5%). FISH localizations demonstrated that the different schemes produced a broader distribution of insertion sites than the pilot screen did (YAN *et al.* 2002). For example, we recovered insertions in the *X* and fourth chromosome centric heterochromatin using these schemes, which were not recovered in our previous study.

Interestingly, the proportion of variegating insertions that are located in centric heterochromatin is much higher than that reported by ROSEMAN *et al.* (1995; 46% centric insertions among variegating lines; the remainder were telomeric), using the same 60F *SUPor-P* insertion in *CyO* as the starting site and a genetic scheme similar to our scheme 1. We previously demonstrated that one reason for the increased recovery of centric insertions in our screens is the use of the more robustly expressed *y* gene as the marker, rather than the *w* gene (YAN *et al.* 2002). In addition, we selected only insertions with a clear all-or-none variegation, which probably accounts for the very high proportion of centric insertions. Insertions displaying altered but not variegated expression ("misexpression" lines) most often mapped to euchromatic sites (YAN *et al.* 2002; this study).

Confirmation of the enrichment for heterochromatic insertions among variegating lines comes from flanking sequences that have been generated for a subset of variegating insertions by inverse PCR and compared to the Release 3 genomic sequence (R. HOSKINS, G. M. RUBIN, R. LEVIS and A. SPRADLING, personal communication, and data not shown). Preliminary analysis confirms that the majority (86%) of variegating insertion flanks with one significant hit to sequences in the genome ($N = 210$) are located in unmapped heterochromatic scaffolds or in heterochromatic regions at the bases of the euchromatic arms (HOSKINS *et al.* 2002). Many lines that have not been localized by FISH were confirmed as heterochromatic by this analysis. These

preliminary results also validated the specific FISH localizations; different insertions located in the same sequence scaffolds mapped by FISH to the same or adjacent cytogenetic bands, with very few exceptions (data not shown). Another 371 lines still need to be localized by FISH, and more flanking sequences need to be determined, but by extrapolation we expect that our collection contains a total of ~560 centric insertions, including the pilot screen lines, and excluding insertions in the balancers. In summary, we conclude that our strategy of isolating heterochromatic insertions by selection for *y* variegation using different mobilization and recovery genotypes has produced a large collection of insertions that will be useful for future studies of heterochromatin structure and function.

Influence of an extra *Y*, a suppressor of PEV, on the transposition of *SUPor-P* into heterochromatin and on the selection of variegating insertions: Zhang and Spradling (1994) have previously shown that selection of *P*-element insertions under conditions where *ry* silencing is suppressed increases the recovery of insertions within centric heterochromatin. We hypothesized that suppression of *y* PEV in the selection generation might similarly increase the yield of variegating insertions by allowing for the recovery of insertions that would otherwise be missed due to the complete silencing of *y*. We also tested whether suppression of PEV during the transposition stage would increase the recovery of variegating insertions, perhaps by making the heterochromatin more accessible to insertion. The results of these experiments are summarized in Figure 1 and Table 1.

Genetic analysis demonstrated that the frequency of variegating insertions increased significantly when an extra *Y* chromosome was present during both the mobilization generation and the selection of transpositions. For the 60F starting site, a 50% increase was seen when both mobilization and recovery occurred in the presence of extra heterochromatin (scheme 5, 18%, Figure 1), in comparison to mobilization and recovery in a normal chromosome constitution (scheme 1, 12%). We also recovered a higher proportion of variegating insertions in *X^Y/X^Y* females in comparison to regular *X/X* females (10% scheme 5 *vs.* 4% scheme 1; Table 2, $P < 0.01$). Similarly, the proportion of recovered variegating insertions was higher in *X^Y/Y* males than in *X/Y* males (scheme 5, 25% *vs.* scheme 1, 19%; $P < 0.05$, Table 2). Recovery of variegating insertions was also significantly higher for the *Y* chromosome starting insertions K13.1 and B783.2 when transpositions were generated and screened in the presence of an extra *Y* chromosome (compare schemes 7 and 8, Figure 1, Tables 2 and 3). A similar but weaker trend in the yield of variegators was observed between schemes 1 and 3 (no extra heterochromatin *vs.* recovery with extra heterochromatin) and schemes 3 and 5 (recovery with extra heterochromatin *vs.* mobilization and recovery with extra heterochromatin; Figure 1, Table 2).

We conclude that an additional *Y* chromosome has a moderate but significant effect on recovery of variegating insertions when present during selection. Most likely, an additional *Y* chromosome also has a weak effect at the mobilization stage, which becomes statistically significant when combined with selection in the presence of an extra *Y*. We propose that the increased recovery in the presence of an extra *Y* results from partial suppression of strong variegating phenotypes that would otherwise be missed (see below). Similarly, the presence of an extra *Y* during mobilization is likely to make other regions of heterochromatin more accessible to insertion.

The effect of additional heterochromatin in the scoring generation in our study appears to be less than that in the studies reported by Zhang and Spradling (1994), which utilized the marker gene ry^+. The significantly higher recovery of variegators in our study, approximately sevenfold over that observed in the *ry* variegation screen, was observed even in the absence of PEV suppression during scoring. Interestingly, we observed a relatively stronger effect of the *Y* chromosome on the recovery of variegators in females (schemes 1 and 5: 4 *vs.* 10%, Table 2). Since *y* variegation in females for individual insertions is more severe than that in sibling males, it is likely that adding a PEV suppressor in this case had a stronger effect on selection of variegators that otherwise would be missed. Thus, it is likely that the level of y^+ gene expression in males is high enough to detect variegation without a suppressor of PEV. We propose that the increased recovery of variegators observed in our studies and the weaker impact of PEV suppression during selection in males are the result of a stronger expression of *y* under silencing conditions, compared to markers such as *ry*.

Gender affects the mobilization of *SUPor-P* into heterochromatin: The proportion of variegating insertions was very low when *SUPor-P* was mobilized in females (compare schemes 1, 3, 5 and 2, 4, 6). Zhang and Spradling (1994) observed the same proportion of variegating insertions when their ry^+ *P* element transposed in males and females (~3%, excluding local transpositions in the *Y* chromosome). However, in their experiments the *P* element was mobilized from a heterochromatic position in females with an extra *Y* chromosome and in males with a regular sex-chromosome constitution. In the results reported here, mobilization from a heterochromatic position in males in the presence of an extra *Y* chromosome substantially increased the yield of variegating insertions. Therefore, it is possible that in the Zhang and Spradling (1994) study transposition in males under similar conditions would have yielded the increased recovery of variegators in males that we observed with *SUPor-P*. Several dominant modifiers of PEV have been shown to be female sterile, while not affecting the male germline (Dorn *et al.* 1986; Reuter *et al.* 1986). We propose that the gender-specific differences in *SUPor-P* insertions into heterochromatic re-

gions reflect a different chromatin organization or nuclear organization of heterochromatin (see below) in the female and male germlines.

Transposition into the heterochromatin is significantly increased if *SUPor-P* transposes from heterochromatic locations: The frequency of transposition depends on the structure of the starting element, but the genomic location of a *P* element does not usually influence the distribution of target sites on nonhomologous chromosomes (Berg and Spradling 1991). Zhang and Spradling (1994) described increased recovery of suppressible ry^+ insertions after mobilization of a silent *Y* chromosome insertion in comparison to a euchromatic starting site. However, only one silent *Y* chromosome insertion was used, and intrachromosomal reinsertions into the *Y* chromosome accounted for over half of the recovered centric insertions.

We tested the generality of the hypothesis that *P* elements located in centric heterochromatin display a preference for remobilization into other heterochromatic sites, using the y^+ marker and eight different *SUPor-P* heterochromatic insertions. Our results suggest that the heterochromatic starting sites significantly influenced the overall transposition rate for both variegators and nonvariegators. Different heterochromatic insertion lines displayed up to sixfold differences in the overall transposition frequency (Table 3). In addition, the presence of an extra *Y* chromosome resulted in a twofold increase in transposition rate for two different *Y* chromosome insertions (Table 3). *P* elements transpose by a "cut-and-paste" mechanism; the process starts with *P*-element transposase-mediated excision of the transposon from the original location (Engels *et al.* 1990; Rio 1990). We propose that differences in mobilization of heterochromatic insertions are associated with different chromatin accessibility of insertion sites for transposase and that accessibility is increased when mobilization occurs in the presence of a strong suppressor of PEV (scheme 7 *vs.* 8, Figure 1 and Table 3).

We also observed a substantial increase in the proportion of variegating insertions recovered in all schemes where centric insertions in the *Y* or *CyO* were used as a source for mobilization (schemes 7, 8, and 9), in comparison to transposition from the 60F euchromatic site (Figure 1). It is remarkable that 25–37% of all insertions recovered after mobilization from heterochromatic sites were variegators, which is very close to the proportion of the genome that is considered to be heterochromatic (Hoskins *et al.* 2002).

The distribution of heterochromatic *SUPor-P* insertions is broad but nonrandom: FISH analysis of 131/502 established variegating lines demonstrated that the insertions recovered in schemes 1–9 significantly extended the coverage of heterochromatic regions in comparison to the 71 centric insertions mapped in the pilot screen (Yan *et al.* 2002). It is possible that the broader distribution resulted from increasing coverage of the 61 heterochromatic bands from approximately one- to approximately threefold. However, some data suggest that different schemes produced different insertion distributions. Although *X* and fourth chromosome insertions are still severely underrepresented in our collection (Tables 1 and 4, Figure 2), we did localize three variegating insertions to the *X* heterochromatin. Interestingly, scheme 8 involved recovery of *X* centric insertions in males and did produce a significantly higher proportion of *X* insertions (Table 4). We hypothesize that the deficit of *X* chromosome centric insertions in most of these schemes could be caused by the greater difficulty of selecting variegating insertions in females, which would carry *X* insertions generated in males. It is possible that this problem was counteracted when mobilization occurred from a *Y* chromosome site (scheme 8) due to physical associations with the *X* chromosome in the germline (see below). In addition, three insertions were localized to the fourth chromosome, and the percentage of fourth chromosome insertions that were recovered in scheme 9 was nearly identical to the proportion of total heterochromatin present in the target chromosomes (Table 4).

The FISH localization and genetic mapping studies also provided information about the impact of different mobilization and selection genotypes and starting sites on the gross- and fine-scale distributions of heterochromatic insertions. First, comparison of mobilization from a second chromosome heterochromatic site (scheme 9) resulted in a distribution of insertions that was proportional to the total amount of heterochromatin in each target chromosome, whereas mobilization from a euchromatic site (scheme 5) in the identical background genotype did not. Second, different heterochromatic starting sites also affected the distribution of new insertions. Mobilizations from *Y* heterochromatin were more likely to insert in the *X^Y*, whereas mobilization from second chromosome heterochromatin produced a more even chromosomal distribution (scheme 7 *vs.* 9, Tables 1 and 4). Third, the presence of an extra *Y* during scoring, and perhaps during mobilization, may act synergistically with the use of a heterochromatic insertion to affect distribution. The nonrandom chromosomal distributions seen in schemes 5 and 1 suggest that the presence of an extra *Y* during mobilization and scoring, which clearly increases the overall frequency of variegating insertions (see above), has little effect on the chromosomal distribution for a euchromatic starting site. However, the relatively even distribution among the heterochromatic bands observed for scheme 5 (compare to the pilot scheme 1, Figure 2) suggests that the fine-scale distribution of insertions is improved by the presence of an extra *Y* for a euchromatic starting site. In addition, the presence of an extra *Y* during selection appears to have an effect specifically on the distribution among the autosomes (scheme 3 *vs.* 1). Selection in the presence of an extra *Y* is likely to "equalize" the ability of insertions in different regions to be selected as *yellow* variegators due to the recovery of insertions that would

otherwise be missed due to low expression. We propose that use of a heterochromatic starting site may further enhance this effect and increase the frequency of *X*, *Y*, and fourth chromosome insertions, as suggested by scheme 9 localizations and the preliminary results from scheme 8 (Table 4). The high number of *X* insertions in scheme 8, despite low numbers of localizations, could reflect an association between the *X* and *Y*, which pair during meiosis (see below).

Models for the effects of genotype and starting site on the recovery and distribution of heterochromatic insertions: Why would mobilization from a heterochromatic site increase centric insertion recovery? In euchromatin or subtelomeric heterochromatin, *P* elements frequently transpose locally *in cis* and to homologous regions of homologous chromosomes *in trans* (Karpen and Spradling 1992; Tower *et al.* 1993; Zhang and Spradling 1993; Tower and Kurapati 1994). This preferential transposition is likely to result from physical proximity of chromosomal regions in germ-cell nuclei (Tower and Kurapati 1994). Zhang and Spradling (1994) have shown that the tendency of *P* elements to transpose locally *in cis* also operates in centric heterochromatin. In our experiments, remobilization of heterochromatic insertions produced a very high frequency of intrachromosomal events leading to a change in the variegation level (up to three times higher than the number of new centric interchromosomal transpositions). We propose that many of these highly frequent events represent local transpositions within heterochromatin that lead to changes in variegation levels.

However, all of the increased recovery of insertions from heterochromatic starting sites reported in Tables 1 and 3 involve interchromosomal events. We propose that the increased recovery of interchromosomal centric insertions from heterochromatic starting sites may be caused by "local" transposition of *P* elements to heterochromatic regions that are closely associated *in trans*. Indeed, we observed a higher proportion of *X^Y* insertions in scheme 7, where *SUPor-P* was mobilized from *Y* chromosome donor sites (44% of variegating insertions, Table 1), compared to scheme 5 (28% of variegating insertions selected among females), where the *SUPor-P* element transposed from 60F. One of the strongest arguments that chromosome associations are involved in the increased frequency of variegating insertions, as opposed to simply mobilization from a heterochromatic site, comes from the observation that only 2% of variegating insertions were in the *X^Y* chromosome after mobilization from second chromosome heterochromatin (scheme 9, Table 1) in comparison to the 44% observed for a *Y* chromosome starting site (scheme 7). Finally, although only six insertions have been FISH localized for scheme 8, two are in the *X* heterochromatin, which in all other schemes has been an extremely inefficient target (Table 4). It is possible that preferential transposition to homologous regions *in trans*, in this case due to pairing of *Y* and *X* chromosomes in the male germline (McKee and Karpen 1990), might operate in heterochromatin.

Several lines of evidence suggest that local transposition to homologs is not the only mechanism leading to the increased frequency of transposition from heterochromatic starting sites to another position in heterochromatin. First, not all *Y* chromosome insertions showed preferential transposition into the *X^Y* chromosome (see Table 3). Second, *Y* insertion B783.3 still showed increased recovery of variegators in scheme 8, despite the absence of the *X^Y* chromosome during mobilization. Third, mobilization of variegating insertions from the second chromosome (scheme 9) resulted in a significant increase in the proportion of *Y* chromosome insertions and a more even distribution among the four target chromosomes, in comparison to scheme 5 (see results). Finally, the proportion of insertions in the *TMS* chromosome is similar in schemes 5 and 9 (see Table 1), suggesting that there is no bias toward second chromosome insertions produced by transposition from second chromosome heterochromatin *vs.* euchromatin. Thus, even if transposition to the homolog might account for a very high proportion of variegating insertions observed for some *Y* chromosome insertions, mobilizations from a heterochromatic donor site cause a general increase in transpositions to heterochromatin and are not restricted predominantly to the homolog. One possibility is that the increased recovery of variegating insertions in schemes 7–9 is caused by the physical proximity of heterochromatic starting sites and target sites in the three-dimensional organization of germline nuclei (Dernburg *et al.* 1996a). Different insertion preferences observed for the *Y* and second chromosome heterochromatic insertions could be explained by preferential associations of different heterochromatic regions between both homologs and nonhomologs.

It is also possible that excised *P* elements are still associated with heterochromatic proteins and prefer to reinsert into centric regions that contain the same proteins, which could be favored due to protein-protein interactions such as homodimerization (Brasher *et al.* 2000; Cowieson *et al.* 2000). Support for this model comes from reports that *P* elements with specific regulatory sequences (*e.g.*, Polycomb responsive elements, or PREs) from the *engrailed* gene and from the *Bithorax* and *Antennapedia* homeotic complexes display a preference for insertion near the chromosomal locus of the gene, a phenomenon known as the "homing" effect (Hama *et al.* 1990; Kassis *et al.* 1992). These transposons, along with transposons that contain regulatory sequences from the *polyhomeotic* gene, also preferentially insert into chromosomal locations containing binding sites for some *Polycomb* group (*Pc*-G) proteins (Kassis *et al.* 1992; Fauvarque and Dura 1993; Chiang *et al.* 1995). These observations suggest that the insertional specificity of *P* elements involves interactions between transgenic and resident PREs, mediated by *Pc*-G proteins (Fauvarque and Dura 1993; Kassis 1994). Considering that both

Pc-G proteins and heterochromatin-specific proteins form repressive chromatin complexes via protein-protein interactions (Pirrotta 1997), it seems reasonable to propose that the homing effect might work in heterochromatin. However, homing due to the presence of general heterochromatic proteins on mobilizing elements cannot account for the strong preference for insertion in the *X^Y* homolog observed in scheme 7.

We propose that similar types and patterns of heterochromatin proteins in source and target regions mediate both the increased frequency and the homolog preferences either by mediating specific physical associations or by homing during mobilization. Associations with homologous chromosomes and specific regions are likely to be preferred, but the general distributions of proteins such as HP1 would also result in a general increase in heterochromatin insertions. We also propose that incorporation of an extra *Y* chromosome in the recovery generation, and perhaps during mobilization, acts to further increase the frequency of recovery of heterochromatic insertions and to broaden the distribution of insertions within the heterochromatin (see below) by acting *in trans* to make heterochromatin chromatin structure more "open" to insertion and expression.

The results of these screens have identified factors that affect the recovery frequencies and distributions of heterochromatic *P* insertions and have created a significant resource for molecular and genetic analysis of Drosophila heterochromatin structure and function. These screens have produced the largest collection of variegating insertions to date, providing approximately ninefold coverage of heterochromatic cytogenetic bands (h1–61). FISH localization of the remaining 371 lines should produce more complete saturation of Drosophila centric heterochromatin, including the *X* and fourth chromosomes, and should help to further define how different mobilization and selection genotypes and mobilization starting sites affect insert distributions. Broader distribution of insertions can also be accomplished by generating more lines using schemes 8 and 9 and by capitalizing on the high frequency of local transposition events, described here and in Zhang and Spradling (1994). This method can be used to saturate any particular heterochromatic region with *P*-element insertions. In addition, flanking DNA sequences are being determined by inverse PCR or cloning (R. Hoskins, G. M. Rubin, R. Levis and A. Spradling, personal communication), and restriction maps can be constructed using single-copy *P* elements as probes (Karpen and Spradling 1992). Information about flanking sequences is being used to create a molecular map of the heterochromatin and to localize unmapped heterochromatic sequence scaffolds (Hoskins *et al.* 2002) to specific cytogenetic regions. These *P* insertions can also be used to identify and characterize heterochromatic genes (Devlin *et al.* 1990; Tulin *et al.* 2002); to examine the behavior of different regions of heterochromatin with respect to inducing PEV, the cell cycle, and replication; and to elucidate the role of heterochromatin in the organization of the interphase nucleus. Current information about individual heterochromatic insertions, including FISH and genomic sequence localizations, can be accessed at http://taputea.lbl.gov/research/het/hetps/.

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