Note

Removal of the Bloom Syndrome DNA Helicase Extends the Utility of Imprecise Transposon Excision for Making Null Mutations in Drosophila

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ABSTRACT

Transposable elements are frequently used in *Drosophila melanogaster* for imprecise excision screens to delete genes of interest. However, these screens are highly variable in the number and size of deletions that are recovered. Here, we show that conducting excision screens in *mus309* mutant flies that lack DmBlm, the Drosophila ortholog of the Bloom syndrome protein, increases the percentage and overall size of flanking deletions recovered after excision of either *P* or *Minos* elements.

TRANSPOSABLE elements have a rich history as mutagenesis tools in *Drosophila melanogaster* (reviewed in RYDER and RUSSELL 2003). Initially, researchers focused their efforts on the use of nonautonomous *P*-element transposons for gene disruption (COOLEY *et al.* 1988). However, *P* elements have insertion biases, preferring to transpose into euchromatic regions, the 5′ regions of genes (TSUBOTA *et al.* 1985; KELLEY *et al.* 1987), and to target sequence motifs similar to the octamer GGCCAGAC (O'HARE and RUBIN 1983). These biases make it unlikely that full genome saturation will be reached using *P*-element mutagenesis. Therefore, mutational systems that utilize transposable elements with different insertion biases have been developed. These include *Hobo* (SMITH *et al.* 1993); the lepidopteran-derived *piggyBac* element, which inserts at TTAA sites (HACKER *et al.* 2003; HORN *et al.* 2003); and *Minos*, a Tc-1/mariner-like element originally isolated from *Drosophila hydei* that inserts at TA dinucleotides (FRANZ and SAVAKIS 1991; LOUKERIS *et al.* 1995). Using a combination of these transposons, the Drosophila Gene Disruption Project has generated inserts in ~60% of the 14,850 annotated genes (SPRADLING *et al.* 1999; BELLEN *et al.* 2004).
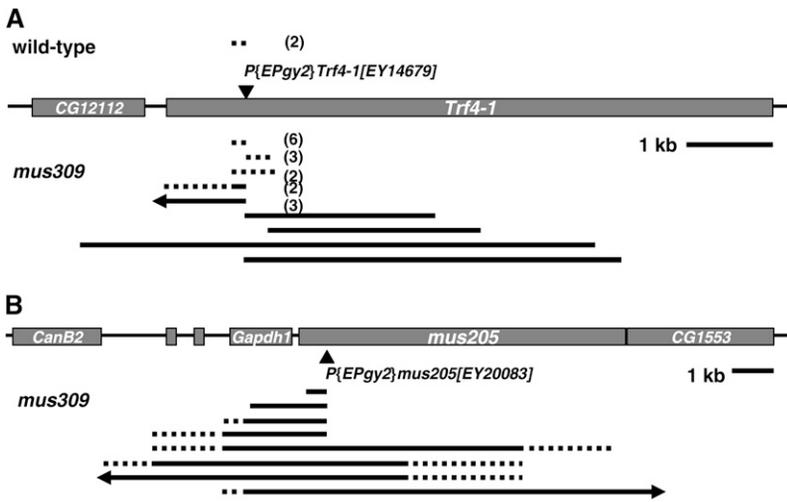
In spite of the growing number of transposon insertions in the Drosophila genome, many are inserted in regions that do not completely abolish gene function, such as 5′-UTRs and introns. This can make it difficult to discern the true null phenotypes of genes. Furthermore, there still exist a sizable number of genes for which no transposon insertions are available. To address these issues, many transposons have been constructed with additional characteristics, such as FRT sites, that make generation of molecularly defined deletions by site-specific recombination relatively straightforward (PARKS *et al.* 2004; THIBAULT *et al.* 2004; RYDER *et al.* 2007). However, until saturation of the genome with these designer transposons is achieved, their utility in creating single-gene deletions remains limited.

A more general approach for generating single-gene deletions that has proven successful is the use of *P* elements in imprecise excision screens. Excision of a *P* element creates a DNA double-strand break with 17 nucleotide noncomplementary ends (BEALL and RIO 1997). If the ends of the break are degraded prior to repair, a deletion of DNA flanking the original insertion site is created (reviewed in HUMMEL and KLAMBT 2008). On average, the frequency of flanking deletions recovered from imprecise excision screens is ~1%. However, this frequency varies tremendously by locus and depends on a multitude of factors that are not well understood, including chromatin structure and local sequence context. Therefore, generation of suitable deletion mutants frequently involves screening many hundreds of independent lines.

An alternative method that uses *P* elements to generate deletions involves screening for events associated with male recombination. These events, which probably arise through a hybrid element insertion mechanism, generate one-sided deletions of sizes ranging from sev-

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FIGURE 1.—Frequency and size of deletions accompanying imprecise *P*-element excision is increased in the *mus309* mutant background. Crosses to generate males possessing both the *P* transposase and the desired *P* element were carried out in bottles containing standard cornmeal-based food at 25°. Excision events occurring in the male premeiotic germlines of flies carrying (A) *P{EPgy2}Trf4-1[EY14679]* and the *P{ry[+], Δ2-3}99B* transposase or (B) *P{EPgy2}mus205[EY20083]* and the *CyO, H{w[+], Δ2-3}* transposase were recovered in male progeny (for *Trf4-1[EY14679]*) or over a deficiency spanning the region (for *mus205[EY20083]*). Only one excision per male germline was analyzed to ensure that all events were independent. Genomic DNA was isolated and subjected to PCR analysis using primers specific to the *P* inverted repeats or to sequences flanking each *P* element. A and B show a genomic region with genes represented as boxes, intergenic regions as lines, and *P* elements as inverted triangles. Deletions were recovered from both wild-type (top panels) and *mus309[D2]/mus309[N1]* mutant males (bottom panels) for *Trf4-1[EY14679]*, while deletions were recovered only from *mus309* mutant males for *mus205[EY20083]*. Solid lines represent confirmed deletions, broken lines represent potential deletions, and arrows represent deletions that extend farther than was tested by PCR. Numbers in parentheses indicate the number of excisions recovered.

eral base pairs to several kilobases (PRESTON and ENGELS 1996). This method, although powerful, involves screening a large number of flies and requires two sequential screens to generate bidirectional deletions.

P-element-induced double-strand breaks are preferentially repaired through homologous recombination using a sister chromatid or a homologous chromosome as a template (ENGELS *et al.* 1990). Previously, we and others have demonstrated that the Drosophila Bloom protein ortholog (DmBlm), a RecQ DNA helicase encoded by *mus309*, is involved in homology-directed repair of these breaks (BEALL and RIO 1996; McVEY *et al.* 2004a). In the absence of DmBlm, repair of a *P*-element-induced break on a plasmid or at a chromosomal locus frequently results in a large, flanking deletion. Several groups have applied this observation to imprecise excision screens using *P* elements and have successfully recovered multiple deletions (ASTROM *et al.* 2003; JOHANSSON *et al.* 2007; Y. RONG, unpublished data). However, a direct comparison between imprecise excision screens carried out in wild-type *vs. mus309* mutant backgrounds has not been published, and little is known regarding the use of this technique with other types of transposable elements. In this study, we used three different transposons to test the hypothesis that the use of a *mus309* mutant background in imprecise excision screens would result in a greater yield of deletions and that these deletions would be larger than those recovered from a wild-type background.

The *mus309* mutant background increases the frequency and size of flanking deletions following *P*-element excision: Previously, we have shown that repair of a double-strand break created by excision of the *P{w[a]}* transposon, located at 13F1–13F4 on the *X* chromosome, is deletion prone in the absence of DmBlm (McVEY *et al.*

2004a). This is likely due to a requirement for DmBlm in D-loop unwinding during homologous recombination (BACHRATI *et al.* 2006; WEINERT and RIO 2007). We have speculated that an unknown endonuclease may cleave D-loops in the absence of DmBlm, resulting in deletions flanking the *P*-element insertion site. To determine whether these observations can be generalized to imprecise excision screens, we tested two additional *P*-element insertions. One of these, *P{EPgy2}Trf4-1[EY14679]*, is inserted within a 1-kb intron of the *Trf4-1* gene on the *X* chromosome (Figure 1A). The other, *P{EPgy2}mus205[EY20083]*, is inserted in a small intron in the *mus205* gene on chromosome *2* (Figure 1B). Both of these *EY* elements contain wild-type copies of the *yellow* and *white* genes (BELLEN *et al.* 2004). Thus, flies possessing *EPgy2* elements have a wild-type body color and pigmented eyes. For our excision screens, we generated males containing the *P* element and a constitutively expressed transposase source, *Δ2-3* (ROBERTSON *et al.* 1988). To test the effect of DmBlm absence, we also conducted the screens in heteroallelic *mus309[D2]/mus309[N1]* mutants (KUSANO *et al.* 2001; McVEY *et al.* 2007).

First, we determined whether loss of DmBlm affected the fertility of males in which *P*-element excision was occurring. We found no significant difference in the percentage of wild-type *vs. mus309* males that were sterile, as defined by an inability of an individual male to produce more than five adult offspring (Table 1). We also quantitated the percentage of males that produced at least one white-eyed excision event. Relative to wild type, we observed a twofold increase in the percentage of *mus309* males that produced at least one white-eyed progeny following *Trf4-1[EY14679]* excision and a 30% decrease following *mus205[EY20083]* excision (Table 1). In both cases, the difference was highly significant (Fisher's

TABLE 1

Fertility and excision rates in wild-type and *mus309* males undergoing transposon remobilization

Insertion	Wild-type: % sterility	*mus309*: % sterility	Wild-type: % of males producing excision	*mus309*: % of males producing excision
Trf4-1[EY14679]	16.5 (97)[a]	15.4 (136)	32.1 (81)	62.6 (115)
mus205[EY20083]	33.3 (108)	31.8 (88)	100 (72)	71.6 (60)
Pvf1[MB01242]	10.4 (113)	9.9 (191)	71.2 (101)	69.7 (172)
dp[MB00453]	40.2 (87)	49.2 (61)	73.0 (52)	87.1 (31)
Tequila[MB00537]	11.3 (53)	14.2 (70)	83.0 (47)	70.0 (60)

Sterility and excision rates were determined for males possessing one copy of the transposon and the corresponding transposase (described in Figures 1 and 2). A male was classified as sterile if it produced fewer than five adult progeny when mated with three or more females.

[a] Numbers in parentheses indicate the number of males tested.

exact test, $P < 0.0001$). Thus, for the two *P* elements tested here, loss of DmBlm had no significant effect on male fertility and had a significant but inconsistent effect on the recovery of excisions in imprecise element screens.

Next, we recovered independent excision events from individual male germlines for further analysis. Most independent excision events that resulted in loss of eye pigmentation also resulted in loss of wild-type body color. However, we did recover some events, mostly from wild-type males, which lost the *white* gene but retained the *yellow* gene, suggesting that an internal *P*-element deletion had occurred. We utilized a PCR strategy to determine the percentage of independently derived excision events that resulted in flanking genomic deletions. Chromosomes with an excision event were recovered in hemizygous males (for *Trf4-1*[EY14679]) or in *trans* to a deficiency spanning the relevant locus (for *mus205*[EY20083]), and genomic DNA was isolated. Primers flanking the *P* insertion site were used in initial reactions to determine whether a precise excision had occurred, as indicated by a PCR product equal in size to that obtained from wild-type flies with no insertion. In cases where no product was observed, we paired a primer complementary to the *P*-element terminal inverted repeats with primers flanking the insertion site in secondary PCR reactions to determine whether any *P* sequence remained. For events in which one or both of the *P*-element ends was missing, additional reactions were performed to determine if unidirectional or bidirectional deletions had occurred. In cases in which we were able to obtain a PCR product spanning the deletion junction, DNA sequencing was performed to determine the exact size of the deletion.

The vast majority of excisions (>95%) obtained in a wild-type background were precise excisions or internally deleted *P* elements. We recovered two deletions (4% of total excisions) of <170 bp from the *Trf4-1*[EY14679] excision in wild-type males, but none following *mus205*[EY20083] excision (Figure 2A). In contrast, *Trf4-1*[EY14679] excision in *mus309* mutants resulted in 20 deletions (28% of total excisions), and *mus205*[EY20083] excision created 8 dele-

tions (20% of total excisions). The minimum size of the deletions obtained in *mus309* mutants varied from tens of base pairs to >10 kb (Figure 2B), and many were bidirectional, extending multiple kilobases in both directions. Of 6 deletions whose exact breakpoints were identified, 3 retained a portion of *P*-element sequence, suggesting that homologous recombination repair initiated but then failed, resulting in a one-sided deletion. The other 3 deletions appeared to involve end-joining repair; 1 deletion had an insertion of 18 nucleotides, suggesting an alternative end-joining process. From these comparisons, we conclude that excision of *P* elements in a *mus309* mutant background increases both the number and the size of flanking genomic deletions relative to excision that occurs in wild-type flies.

Absence of DmBlm also increases the yield of large deletions following imprecise excision of *Minos* elements: Recently, METAXAKIS *et al.* (2005) demonstrated that remobilization of *Minos* transposons can also be used to produce deletions adjacent to the original insertion site. However, the proportion of deletions recovered relative to total excisions was small, and the largest confirmed deletion was only 800 bp. Approximately 25% of *Minos*-induced double-strand breaks in females heterozygous for the insertion are repaired by nonhomologous end joining and mismatch repair, frequently resulting in a 6-bp insertion, or "footprint," relative to the original target sequence (ARCA *et al.* 1997). The other 75% are likely repaired by homology-directed repair.

Because DmBlm is required to prevent deletions during homologous recombination, we tested whether imprecise excision of *Minos* in flies lacking DmBlm would also result in an increased probability of recovering large deletions in nearby sequence. Males containing the *Minos* transposase driven by a heat-shock promoter and *Mi{ET1}* insertions on chromosomes *X, 2,* and *3* (located in the *Pvf1, dp,* and *Tequila* genes, respectively) were generated (Figure 3). To test the effects of DmBlm loss, the *mus309*[D2] and *mus309*[N1] alleles were used in combination with the *Pvf1*[MB01242] and *dp*[MB00453] insertions, and the *mus309*[D2] and *mus309*[D3] alleles (KUSANO *et al.* 2001) were used with the *Tequila*[MB00537] insertion (the *mus309*[D3]
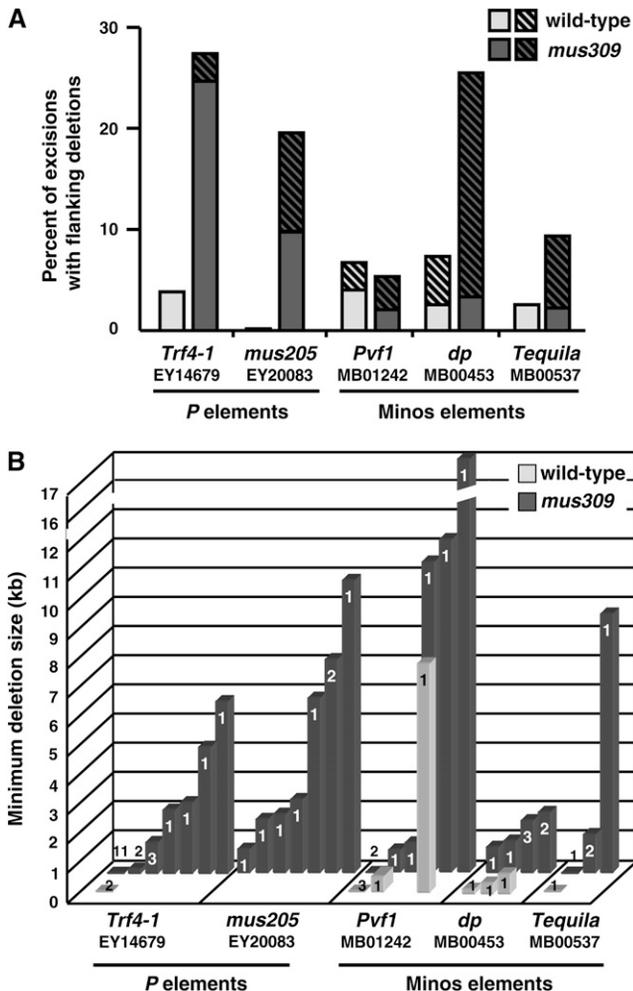
FIGURE 2.—Number and size of deletions following transposon excision is increased in *mus309* mutants. (A) Histogram showing the percentage of excisions accompanied by flanking deletions in wild-type and *mus309* mutants. Solid bars indicate unidirectional deletions; hatched bars indicate bidirectional deletions. (B) Histogram showing the minimum size of deletions, as determined by the absence of a PCR product, in wild-type and *mus309* mutants.
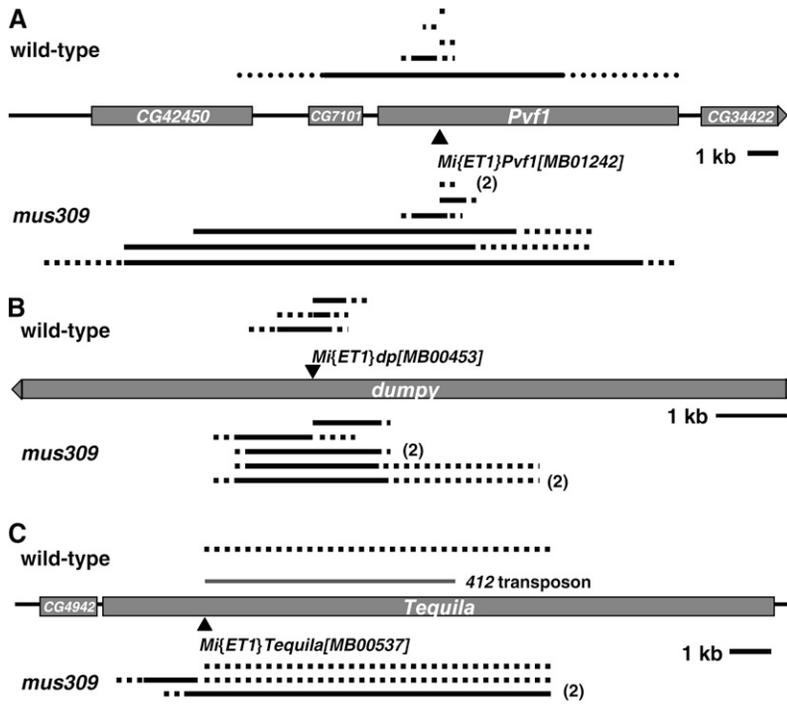
allele was crossed onto the *Tequila^MB00537*-bearing chromosome by standard genetic methods). For these three screens, we compared *mus309* heteroallelic males to *mus309* heterozygous males. Because *mus309* heterozygotes behave as wild types in double-strand break repair assays (McVey *et al.* 2007), we will hereafter refer to them as wild type.

Similar to what we observed in the *P*-element screens, loss of DmBlm had no significant effect on the percentage of males that were sterile (Table 1). Because *MB* elements contain the EGFP gene, we identified excision events by screening for loss of green fluorescence in the progeny from fertile males. The absence of DmBlm did not affect the percentage of males that produced at least one *Minos* excision event (Table 1). Therefore, neither fertility nor excision rate is negatively impacted in the absence of DmBlm for *Minos* transposons.

We isolated independent *Minos* excision events and used PCR analysis similar to that described for the *P*-element screens to determine the number and size of deletions in wild-type and *mus309* mutants. We observed that the frequencies of precise excision or end-joining repair resulting in a 6-bp footprint were approximately equal between wild-type and *mus309* mutant males (data not shown). Interestingly, excisions isolated from wild-type males were more likely to involve internal deletions of *Minos* sequence, whereas an increased percentage of excisions occurring in *mus309* mutants had deletions of flanking genomic sequence. For the *dp^MB00453* and *Tequila^MB00537* insertions, the percentage of genomic deletions from *mus309* mutant males was 26% and 9%, respectively, compared to 8% and 3%, respectively, for the *mus309* heterozygous controls (Figure 2A). The percentage of genomic deletions that resulted from repair following *Pvf1^MB01242* excision was similar for *mus309* heteroallelic and heterozygous males (6% *vs.* 7%). These data are consistent with the model that double-strand breaks created by *Minos* excision can be repaired either by nonhomologous end joining or by homologous recombination and that DmBlm is required for efficient gap repair during homologous recombination.

For all three *Minos* insertions, the size of deletions was also increased when recovered from *mus309* mutants (Figure 2B). Of 9 deletions isolated from wild type, only 1 (11%) had a minimum size >1 kb. In contrast, 11 of 18 deletions (61%) isolated in a *mus309* mutant background had a deletion >1 kb, and 4 of 18 (22%) involved deletions of at least 8 kb. During the process of PCR mapping of the deletion breakpoints for the *dp^MB00453* and *Tequila^MB00537* excisions, we became aware of the existence of a highly repetitive sequence and an endogenous *412* transposon to one side of each of these respective *Minos* elements. This impaired our fine-scale mapping and may have caused us to underestimate the minimum size of several of the deletions obtained from *mus309* mutants. Notably, the percentage of bidirectional deletions relative to total deletions was also increased for all *Minos* insertions in the *mus309* mutants (71%) compared to wild type (44%).

Loss of DmBlm does not promote deletion formation following *piggyBac* excision: *PiggyBac* elements have also been utilized in genomewide transposon saturation screens (Thibault *et al.* 2004). However, no reports of imprecise excision of *piggyBac* elements have been published, preventing their use in traditional deletion screens. To formally test whether imprecise excision of *piggyBac* elements can occur in either wild-type or *mus309* mutants, we conducted screens with three different *piggyBac* elements—*PBac{RB}WRNexo^04496*, *PBac{RB}CG6719^00315*, and *PBac{PB}lig3^c03514*—in males that also inherited a constitutively expressed *piggyBac* transposase under the control of the α*Tub84B* promoter. Overall, we found that germline excisions with *piggyBac* were less frequent than with either *P* or *Minos*

FIGURE 3.—Frequency and size of deletions accompanying imprecise *Minos* excision is increased in the *mus309* mutant background. Crosses to generate males possessing both the *Minos* transposase and each *Minos* element were done in bottles containing standard cornmeal-based food at 25°. Parental flies were moved to new bottles every 2 days for three consecutive broods. To induce transposase expression, cleared bottles were heat-shocked for one hour in a 37° incubator every day until adults eclosed. Excision events occurring in male premeiotic germlines of flies carrying the (A) *Mi{ET1}Pvf1[MB01242]*, (B) *Mi{ET1}dp[MB00453]*, or (C) *Mi{ET1}Tequila[MB00537]* transposons, together with the *SM6a,P{hsMi\T}2.4* transposase, were recovered in male progeny (for *Pvf1[MB01242]*), over a deficiency (for *Tequila[MB00537]*), or in homozygotes (for *dp[MB00453]*). Genomic DNA was isolated for independent excisions and analyzed by PCR using primers specific to *Minos* or to sequences flanking each insertion. Genomic regions with genes are represented as boxes, intergenic regions as lines, and *Minos* elements as triangles. The *412* endogenous retrotransposon is located adjacent to *Tequila[MB00537]*. For all three insertions, deletions were recovered from both wild-type (top panels) and *mus309* mutant males (bottom panels). Solid lines represent confirmed deletions, broken lines represent potential deletions, and arrows represent deletions that extend farther than was tested by PCR. Numbers in parentheses indicate the number of excisions recovered.

elements. We obtained 25 excisions from a wild-type background using *PBac{RB}WRNexo[e04496]*, all of which were precise. When *mus309[D2]/mus309[N1]* males were used, we recovered 64 excision events from 25 independent male germlines, only 1 of which was imprecise. This single inaccurate repair event deleted 12 bp directly adjacent to the insertion site. Screens using an alternative *piggyBac* transposase source driven by the *Hsp70* promoter or conducted in *mus309* heterozygous females were also unsuccessful in generating any imprecise excisions (data not shown). In addition, no imprecise excisions were obtained from wild-type or *mus309* mutant males with the *PBac{RB}CG6719[e00315]* or *PBac{PB}lig3[e03514]* elements (data not shown). We conclude that the absence of DmBlm does not appreciably improve the yield of imprecise excisions or deletions for *piggyBac* elements.

PiggyBac is the first example of a DDE superfamily transposon in eukaryotes (MITRA *et al.* 2008). Similar to bacterial *Tn5* and *Tn10, piggyBac* transposition involves a transposon hairpin intermediate that is subsequently cleaved, producing four-nucleotide TTAA overhangs on the 5′-ends of both the transposon and the donor DNA. These clean breaks can be easily repaired by nonhomologous end joining. In contrast, the complementary-ended breaks created by the I-*Sce*I endonuclease, which creates 3′ TTAT overhangs, are frequently repaired inaccurately in Drosophila (PRESTON *et al.* 2006). Therefore, it seems likely that the *piggyBac* transposase itself may promote accurate rejoining of the double-strand

break created during transposition and may prevent other repair pathways, such as homologous recombination, from acting upon the break.

A general strategy for the use of double-strand break repair mutants to create genomic deletions: DNA double-strand breaks in *D. melanogaster* can be repaired by multiple pathways, including homologous recombination, single-strand annealing, nonhomologous end joining requiring DNA ligase IV, and DNA ligase IV-independent alternative end joining (PRESTON *et al.* 2006). These four pathways are not mutually exclusive and can compensate for each other if one is disabled. Our data obtained with *P* and *Minos* elements suggest that, in the absence of DmBlm, homologous recombination is impaired and break repair proceeds through a deletion-prone alternative end-joining pathway. Similarly, several groups have shown that repair of double-strand breaks created by the I-*Sce*I endonuclease in the absence of Drosophila DNA ligase IV also causes an increase in flanking deletions (PRESTON *et al.* 2006; WEI and RONG 2007). We have not observed any difference in deletion frequency during imprecise excision screens of *P* elements conducted in wild-type *vs.* *lig4* mutant backgrounds (MCVEY *et al.* 2004B). However, we have not systematically tested the use of a *lig4* mutant background for *piggyBac* or *Minos* excision screens.

Zinc-finger nucleases (ZFNs) have recently emerged as an effective way to induce double-strand breaks in a number of eukaryotic organisms, including Drosophila, *Arabidopsis thaliana, Caenorhabditis elegans,* and *Danio*

rerio (Bibikova *et al.* 2002; Lloyd *et al.* 2005; Morton *et al.* 2006; Carroll *et al.* 2008; Doyon *et al.* 2008; Meng *et al.* 2008). By utilizing ZFNs in mutants lacking one or more critical components of the different repair pathways, it is possible to bias repair of site-specific breaks toward a desired outcome. For example, inducing breaks in the absence of DNA ligase IV increases the proportion that are accurately repaired by homologous recombination in both Drosophila and *C. elegans* (Morton *et al.* 2006; Bozas *et al.* 2009). In contrast, loss of both Rad51 and DNA ligase IV causes a majority of ZFN-induced breaks to be repaired by deletion-prone alternative end-joining pathways (Bozas *et al.* 2009). It will be interesting to determine whether mutation of *mus309* similarly increases inaccurate repair and causes large deletions when ZFNs are used as a mutagenic agent.

Although the use of transposons to induce genomic deletions is a powerful tool for Drosophila geneticists, transposition can occasionally create second-site mutations that may affect subsequent phenotypic analysis. This might be of particular concern in a *mus309* mutant background, which causes elevated genomic instability in the form of mitotic crossovers (McVey *et al.* 2007). In a separate study, we have used a *lacZ* reporter system (Garcia *et al.* 2007) to measure the spontaneous mutation frequency in *mus309* mutants. We find that the overall point mutation frequency is unchanged relative to wild-type flies, while the frequency of genomic rearrangements (deletions, inversions, and translocations) is elevated approximately twofold (A. Garcia, M. Lundell, J. Vijg and M. McVey, unpublished results). These genomic rearrangements are likely a result of the inaccurate repair of endogenous double-strand breaks. Although these data suggest that the probability of a second-site mutation following *P*-element excision may be slightly elevated in *mus309* mutants, such events can easily be discerned by comparing the phenotypes of multiple independent excisions or by transgenic rescue.

Conclusions: The goal of imprecise transposon excision screens is to create deletions that remove genes or regions of genomic sequence. The studies presented here demonstrate that performing screens with *P* and *Minos* insertions in male flies lacking DmBlm improves the chances of obtaining multiple large deletions. This approach does not affect male fertility or overall recovery of germline excisions. Furthermore, by utilizing the *mus309^{N2}* allele, which is female fertile but associated with deletion-prone repair of breaks (McVey *et al.* 2007), the technique can also be applied to imprecise excision screens in females. We anticipate that this approach will benefit researchers working with Drosophila (and perhaps other model organisms) by significantly reducing the amount of labor required to obtain null alleles of genes for which transposons are inserted far from coding sequences.

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