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Super-sized deletions

Improved transposon excision screens using a *mus309* mutant background

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Over the past two decades, a large collection of transposable elements inserted at various locations in the *Drosophila melanogaster* genome has been assembled. These transposons are frequently utilized in imprecise excision screens to generate deletions in genes of interest. In general, these screens involve genetic manipulations to combine a non-autonomous transposon and the appropriate transposase in individual male or female flies. DNA double-strand breaks are created via transposase action in both somatic and germline cells of these individuals and inaccurate repair events are recovered in the progeny. Because deletion-prone repair of transposon-induced double-strand breaks is rare, these screens generally require a significant investment of time and resources. We recently reported that conducting imprecise excision screens in *mus309* mutant flies, which lack the Drosophila ortholog of the Bloom Syndrome helicase, results in an increase in both the number and size of deletions recovered. Here, we provide additional information for Drosophila researchers wishing to utilize this technique. In addition, we discuss how the general principle behind this technique can be applied in other contexts where double-strand breaks are being generated for the purpose of genome modification.

Key words: transposons, *P* element, *minos* element, double-strand break repair, bloom syndrome, helicase

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Transposable elements have long been a staple of mutagenesis screens in Drosophila. *P* elements, in particular, have been used as tools in attempts to saturate the genome with insertions in all annotated genes.[1-3] Recently, several groups have engineered *piggyBac* and *Minos* elements for use in mutagenesis screens,[4-6] greatly increasing the number of genes disrupted by transposons. Current estimates suggest that over 60% of all protein coding genes contain at least one transposon insertion. However, many of these insertions are located within non-coding regions of genes, including 5' and 3' untranslated regions and introns. Such insertions may still allow for residual gene expression, resulting in hypomorphic or neomorphic mutant alleles. In cases where a true null phenotype is desirable, deletion alleles may be required.

Fortunately, *P* elements can also be mobilized to create large deletions removing one or more flanking genes. *P* transposase makes dual incisions at the inverted repeats of a *P* element, thereby creating a DNA double-strand break at the original insertion site.[7] The resulting break possesses dual 3' single-stranded DNA ends, each of which is 17 nucleotides long. These breaks are normally repaired through homologous recombination, using either the sister chromatid or, less frequently, a homologous chromosome as a template.[8,9] Alternatively, end-joining mechanisms can repair the lesion, although this is a fairly rare occurrence in most contexts.

The majority of products following repair of a *P* element-induced break can be assigned into one of four classes: fully restored *P* elements, internally deleted *P* elements, precise excisions, or repair events that delete DNA sequences to one or both sides of the insertion site. The latter three classes frequently result in the loss of a dominant marker gene such as *white* or GFP; marker loss can therefore be used to screen for excisions. Most repair events isolated in excision screens

Table 1. Frequencies and sizes of deletions isolated from imprecise excision screens

Transposon	Wild-type deletion frequency[a]	*mus309* deletion frequency	Wild-type average minimum deletion size in base pairs[b]	*mus309* average minimum deletion size in base pairs
		***P* elements**		
P{EPgy2}Trf4-1^{EY14679}	2.0% (97)	14.7% (136)	10 (2)	933 (20)
P{EPgy2}mus205^{EY20083}	0% (108)	9.1% (88)	-	4685 (8)
P{EPgy2}DNApol-η^{EY07711}	1.3% (230)	N.D.	1438 (3)	N.D.
P{EPgy2}mus309^{EY03745}	0.5% (765)	N.D.	1600 (4)	N.D.
		***Minos* elements**		
Mi{ET1}PvfI^{MB01242}	4.4% (113)	3.7% (191)	1673 (5)	5814 (7)
Mi{ET1}dp^{MB00453}	3.4% (87)	11.5% (61)	440 (3)	1650 (7)
Mi{ET1}Tequila^{MB00537}	1.9% (53)	5.7% (70)	10 (1)	2868 (4)

[a]Deletion frequencies were calculated as the percentage of males who produced at least one individual with a deletion removing genomic sequence to one or both sides of the original transposon insertion site. Numbers in parentheses indicate the total number of individual males assayed. [b]The minimum deletion size for each event was determined through a combination of PCR analysis and DNA sequencing across deletion junctions. In many cases, the exact size of the deletions could not be determined, due to repetitive sequences adjacent to the transposon. In these cases, the reported minimum deletion size is likely an underestimate. Numbers in parentheses indicate the total number of deletions analyzed. N.D. indicates no data were collected.

(95–99%) are either precise excisions (involving repair from a homolog) or internally deleted *P* elements (resulting from incomplete homologous recombination followed by end joining). Imprecise excisions that involve flanking deletions are rare and deletions that do occur are usually small (less than one kilobase). In cases where the original insertion is located in a large intron or 5' UTR, or when an excision event that removes an entire gene is desired, the recovery of a suitable deletion may involve screening hundreds of independent excisions. In addition, since the phenotype of a null mutation is usually unknown, the screening protocol requires that each independent excision event be tested by PCR. Thus, carrying out an imprecise excision screen can be a time- and labor-intensive process. Various modifications to transposon mobilization screens have been utilized to increase the yield of large deletions,[10,11] but these also vary in their efficiency and frequently require specially constructed stocks.

We recently described a modification to the screening protocol which results in a dramatic increase in both the percentage and overall size of deletions recovered following transposon mobilization.[12] Our strategy is based on several reports suggesting that double-strand break repair in the absence of the Drosophila Bloom Syndrome helicase (encoded by the *mutagen sensitive 309*, or *mus309* gene) frequently produces repair products accompanied by large deletions.[13-16] We formally tested this using multiple different *P*, *piggyBac* and *Minos* transposons located at various positions in the genome. Specifically, we mobilized each of these transposons in wild-type and *mus309* mutant males and determined the percentage of individuals that produced at least one fly possessing a deletion flanking the original insertion site (**Table 1**). We then estimated the minimum size of these deletions in each genetic background. We found that the overall frequency of deletion-generating repair events following excision of *P* elements in a *mus309* mutant background was 12.5%, compared to 0.8% percent in a wild-type background. Furthermore, the average minimum deletion size was 2,000 base pairs in *mus309* mutants, compared to 1,190 base pairs for wild-type flies.

Similarly, conducting *Minos* excision screens in a *mus309* mutant background increased both the efficiency of deletion recovery and the average deletion size. For three different *Minos* insertions, the overall frequency of deletions recovered from *mus309* and wild-type screens was 5.6% and 3.6%, respectively, while the average minimum deletion size was 3,540 base pairs for *mus309* mutants, compared to 1,080 base pairs for wild-type. We were unable to recover deletions following mobilization of *piggyBac* elements in wild-type or *mus309* mutant flies, consistent with a previous report.[17]

For the screens described above, we utilized three different *mus309* mutations: *D2*, a nonsense mutation; *D3*, an E→K missense mutation in the conserved DEAH helicase domain, and *N1*, a deletion that removes the N-terminus and part of the helicase domain of DmBlm.[18,19] These three alleles behave as null mutations in double-strand break repair assays, are female sterile due to maternal effect embryonic lethality, and are male fertile.[19] The *D2* and *D3* chromosomes are homozygous lethal, likely due to the presence of second-site mutations. Therefore, we have found that it is convenient to create heteroallelic combinations of *mus309* mutations for screening purposes, such as *D2/N1* or *D2/D3*. Based on a previous report that *mus309*^{D3} heterozygotes also have a measurable defect in homologous recombination repair of *P* element-induced breaks,[19] we have performed pilot tests to determine whether deletion frequencies are also elevated in *D3* heterozygotes. To date, we have not observed any difference in deletion frequencies between wild-type and *D3* heterozygous males, suggesting that it is necessary to inactivate both copies of *mus309* to significantly increase the efficiency of deletion recovery.

We have also conducted double-strand break repair experiments with the *mus309*^{N2} allele, an in-frame N-terminal

deletion.[19] *N2* behaves as a null allele in double-strand break repair assays, but is partially female fertile. Therefore, it can be used in screens in which isolation of excision events from the female germline are desired. All of these *mus309* alleles are currently available from the Bloomington Stock Center (**Table 2**). The *D2* and *N2* alleles have been recombined onto chromosomes bearing the *Delta2-3 P* transposase source, simplifying the generation of stocks for imprecise *P* excision screens. In addition, the heat-shock inducible *Minos* transposase source, located on a second chromosome balancer,[6] is now available from the Bloomington Stock Center.

At present, the mechanism responsible for deletion-prone repair in flies lacking the DmBlm helicase is poorly understood. In vitro, DmBlm can unwind displacement loop (D-loop) intermediates that form during homologous recombination,[20] suggesting that the deletions in *mus309* mutants may result from a defect in homologous recombination repair. However, *rad51* mutants do not have an increased rate of repair-associated deletions,[21] implying that the defect in *mus309* mutants occurs after Rad51-mediated strand invasion. To explain this finding, we have proposed that an unknown endonuclease cleaves irresolvable D-loop structures, resulting in a loss of sequence flanking the original break site.[22] Further cycles of strand invasion, D-loop formation, and cleavage can result in extensive deletions. Alternatively, the deletions may result from exonuclease activity and end joining following D-loop disassembly.

Regardless of the actual mechanism of deletion formation, these models imply that any double-strand break capable of being repaired by homologous recombination can be used to generate genomic deletions, and that these deletions will be larger and more abundant in *mus309* mutants. Therefore, the *Minos* element, which upon excision produces a double-strand break that can be repaired through both homologous recombination and end-joining mechanisms,[23] can be successfully employed in deletion screens. In contrast, *piggyBac* elements create breaks that appear to be refractory to homologous recombination repair and are therefore almost always repaired accurately, perhaps through a process mediated by the *piggyBac* transposase itself. An important corollary of this principle is that double-strand breaks induced by zinc finger nucleases should be preferentially repaired by deletion-prone processes in *mus309* mutants. This could easily be tested by injecting RNA encoding zinc finger nucleases targeting a gene with a visible mutant phenotype[24] into *mus309*^{N2} mutant embryos and comparing the frequency of mutants recovered from *N2* mutants vs. wild-type flies.

Theoretically, it should also be possible to generate flanking deletions following transposon mobilization by interfering with other double-strand break repair pathways. As an example, two groups have observed increased deletion frequencies after induction of DNA breaks by the I-*Sce*I endonuclease in flies lacking DNA ligase IV, which is required for canonical non-homologous end joining.[13,25] However, we have not observed any measurable effect on recovery of *P* element-induced deletions in *lig4* mutant flies.[26] One possible explanation for this could be that *P* element-induced breaks are preferentially repaired by homology-directed repair pathways. Notably, we have recently found that repair of double-strand breaks following *P* element mobilization in Drosophila *mus308* mutants is also deletion prone, suggesting that there are multiple mutant backgrounds which can be utilized to increase the yield of deletions obtained from transposon excision screens.

As a final note, there are certain factors that must be considered when conducting excision screens in *mus309* mutants. First, males lacking DmBlm have heightened mitotic recombination[19] and approximately a two-fold increase in gross chromosomal rearrangements (Garcia A, Lundell M, Vijg J and McVey M, unpublished results). Therefore, as with any screen, proper controls must be performed to demonstrate that any mutant phenotype is associated with the deletion and not with a secondary mutation. Second, if the transposon to be mobilized is located on the third chromosome, the insertion must first be recombined onto a chromosome bearing a *mus309* mutation, and the *mus309* mutation must be subsequently removed by recombination once the desired deletion has been obtained. The identification of additional repair genes on other chromosomes that normally act to prevent deletion formation will eliminate this inconvenience. In summary, we propose that Drosophila researchers utilizing either *P* or *Minos* elements in deletion screens will significantly benefit by conducting their screens in a *mus309* mutant background.

Table 2. Useful stocks for conducting imprecise excision screens in the *mus309* mutant background

Bloomington stock center number	Genotype
8656	*mus309[D3] ry[506]/TM3, Sb[1] ry[RK]*
8657	*st[1] mus309[D2] Sb[1] P{ry[+t7.2] = Delta2-3}99B/TM6B, Tb[1]*
28878	*w[1118]; mus309[N1]/TM3, Sb[1]*
28879	*y[1]/Dp(1;Y)y[+]; mus309[N2] ry[506] Sb[1] P{ry[+t7.2] = Delta2-3}99B/ TM6B, Tb[1]*
24613	*w[1118]; noc[Sco]/SM6a, P{w[+mC]=hsILMiT}2.4*

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