Targeted gene replacement by homologous recombination in Drosophila stimulates production of second-site mutations

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Targeted gene replacement by homologous recombination in Drosophila stimulates production of second-site mutations

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Key words: Drosophila, BEAF, insulator, gene targeting, homologous recombination

Introduction

Gene targeting by homologous recombination allows specific mutations to be introduced into genes of interest. Since the development of this method in Drosophila in 2000, it has rapidly been adopted by researchers as an important part of their toolkit for generating mutations in flies.1 To carry out gene targeting by the ends-in method in Drosophila, a donor construct carrying mutated DNA from the gene to be targeted is randomly inserted into the genome by P-element-mediated transformation (Fig. 1). Then, a site-specific recombinase (FLP) and a site-specific endonuclease (I-Sce1) are used to excise the mutated transgene as an extra-chromosomal DNA molecule that carries a double-stranded break (DSB). The presence of the DSB stimulates homologous recombination between the excised donor and the homologous chromosomal target locus.1,2 The desired fate of an ends-in targeting molecule is integration into the gene of interest, producing a tandem duplication of the targeted gene with a marker gene between the copies. The target locus duplication can be reduced to a single copy by homologous recombination between the repeated sequences, which also deletes the marker gene. This event is stimulated by an I-CreI-generated DSB between the repeats.3

While using this technique to generate mutations in the BEAF gene,4 we obtained second-site lethal mutations at unknown locations on target chromosomes. One lethal second-site mutation was apparently introduced at the same time as the gene targeting event (the FLP, I-Sce1 step), while the other was introduced during reduction of the gene duplication to a single copy (the I-CreI step). These unexpected mutations complicated the analysis of the BEAF mutations until they were discovered and removed (Fig. 2). Because the two chromosomes we isolated that lacked a wild-type BEAF gene both had second-site lethal mutations, this raised the question of whether the method of isolating mutations by gene targeting stimulates the production of non-targeted lethal mutations. This question is addressed in this report.

Results

Determining lethal second-site mutation rates. The protocol for gene targeting by homologous recombination1-3 was followed to determine the rates of occurrence of lethal second-site mutations. We used an isogenized third chromosome marked with a P[w+ mus30J] P-element4 as the reporter, which we refer to as 3 iso (Materials and Methods). Five schemes were used, as illustrated in Figure 3. In all cases, 3 iso males were crossed to appropriate females (F0 cross) and the larvae were heat shocked for one hour at 37°C. In the two experimental strategies, the starting females had a mutant BEAF “donor” transgene (mBF) on the X chromosome and transgenes encoding either heat shock-inducible FLP and I-SceI transgenes or a heat shock-inducible I-CreI transgene. The mBF transgene has recognition sites for FLP, I-Sce1 and I-Cre1, and was previously used to generate mutations in the BEAF gene on the second chromosome by homologous recombination.5 For the negative control, the starting females lacked mBF, FLP, I-Sce1 and I-Cre1 transgenes. For the “no donor” controls, the starting females lacked the mBF transgene but had either the FLP and I-Sce1 transgenes or the I-Cre1 transgene. Single males were used in the F1 cross to allow subsequent determination of the number of 3 iso chromosomes that picked up a lethal mutation. Following the cross strategies outlined in Figure 3, the chromosomes with

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the mBF, FRT, I-SceI and I-CreI transgenes were removed leaving flies with 3 iso from a single male balanced over a TM3 chromosome. These 3 iso/TM3 flies were self-crossed, and if all flies in the next generation had TM3 then 3 iso had picked up a lethal mutation.

Results of these crosses are presented in Table 2 and Supplementary Tables A and B. We found a spontaneous lethal mutation rate of 2.95% in the negative control. Others have reported spontaneous mutation rates of around 0.6%.7,8 Our rate might be higher because of the strains we were using, and it might have been stimulated by the heat shock.9 A chi-square analysis indicated that the lethal mutation rates in the FLP, I-SceI and the I-CreI "no donor" controls were not significantly higher. However, the roughly three-fold higher mutation rate for flies with the mBF, FLP and I-SceI transgenes was significant at a confidence level of nearly 95%, and the roughly 3.5-fold higher mutation rate for flies with the mBF and I-CreI transgenes was significant at a confidence level of nearly 99%. Perhaps activation of DNA repair pathways by the double-strand breaks present during these processes led to chromosomal instability at other locations.10,11 It is also possible that the I-SceI and I-CreI endonucleases acting at suboptimal recognition sites in the genome contributed. Regardless of the mechanism involved, our results indicate that expressing these endonucleases in flies with perfect cut sites for these enzymes leads to a modest but significant increase in the rate at which non-targeted mutations arise.

In addition to the lethal mutations described above we also obtained unexpected recombination products (Table 1). This was discovered during the molecular characterization of these products.6 Unexpected recombination products after gene targeting have been reported by other Drosophila researchers, such as during the generation of Su(var)3-7 mutations12 and Nap1 mutations.13 Additionally, insertions or deletions have been found after some gene targeting events, such as at the yellow,1 Su(var)3-7,12 and pug and CG11305 genes.3 Although the various products

Figure 1. Shows the process of gene targeting event by homologous recombination using the ends-in method. A donor construct carrying mutated DNA from the gene to be targeted is randomly inserted into the genome by P-element-mediated transformation. Then, a site-specific recombinase (FLP) and a site-specific endonuclease (I-SceI) are used to excise the mutated transgene as an extra-chromosomal DNA molecule that carries a double-stranded break (DSB). The presence of the DSB stimulates homologous recombination between the excised donor and the homologous chromosomal target locus. The desired fate of an ends-in targeting molecule is integration into the gene of interest, producing a tandem duplication of the targeted gene with a marker gene between the copies. (A, B & AB = represent the sites at which point mutations (*) are inserted into the donor construct, B = BglII restriction enzyme cut site, US5/US3 and DS5/DS3 = upstream and downstream PCR primers respectively).
by wild-type transgenes, and three of these four only reported experiments done with the mutant chromosome in a homozygous state. They did not combine mutant alleles with each other or with an appropriate chromosomal deficiency (which would keep second-site mutations heterozygous). Because of this plus our experience with this method, we feel it is important to raise awareness of the necessity to use care when analyzing mutations generated by gene targeting.

**Discussion**

Gene targeting by homologous recombination in Drosophila is a valuable tool. However, there is a distinct possibility that second-site mutations are often introduced into chromosomes.
when using this technique despite the expectation that only targeted mutagenesis will occur. Although this possibility is not addressed in many publications reporting use of this technique, detecting and eliminating second-site mutations was essential for the accurate analysis of BEAF mutations. While the mechanism responsible for introducing the second-site mutations remains uncharacterized, our results and those of others highlight the need for care in working with mutations generated using homologous recombination.

Here in this paper we carried out the gene targeting method in experimental lines using a mutant BEAF transgene. Our goal was to measure the rate of production of lethal mutations on a non-targeted chromosome in the presence and absence of the I-Crel or I-Scel FLP transgenes, with and without a transgene containing recognition sites for these enzymes. Our results clearly indicate a significant increase in the rate of formation of non-targeted or second-site lethal mutations in the experimental lines.

As with other mutagenesis methods, well-established techniques such as backcrossing, mapping, complementation with a wild-type transgene, and the use of multiple independently derived alleles must be used to verify that observed phenotypes are attributable to the mutation of interest. Bearing this potential complication in mind, gene targeting is a powerful technique that allows the generation of mutant alleles that would otherwise be difficult to obtain.

**Materials and Methods**

**Isogenizing the third chromosome.** The third chromosome used in these crosses must be free of any lethal mutations. For this purpose it was isogenized. The third chromosome we selected is marked by a P[w\'* mus301\'] transgene. The w’ confers orange eye color. Homozygous males of the P[w\'* mus301\']/P[w\'* mus301\'] genotype were crossed with TM3/ET50 females. Male progeny of the genotype P[w\'* mus301\']/TM3 emerging from this cross were individually crossed to TM3/ET50 females again. Progeny flies of the genotype P[w\'* mus301\']/TM3 were then self-crossed, and flies homozygous for P[w\'* mus301\']/P[w\'* mus301\'] (we refer as 3 iso) from these vials were then self-crossed and maintained as a stable line.

**Fly crosses.** Five cross schemes were adopted. The first three crosses gave rise to the control lines and remaining two crosses generated the experimental lines.

Negative control line: This control line lacks the mutant BEAF transgene (P[w\'* mBF\']) on the X chromosome and transgenes for any of the recombinases (I-Crel or I-Scel or FLP). 3 iso males were crossed with TM3/ET50 virgin females. The vials were emptied after 3 days and progeny larvae were heat shocked for 1 hr at 37°C in a water-bath. Adult P[w\'* mus301\']/TM3 males eclosing from these vials were then crossed individually with TM3/ET50 females. By this stage ~250 vials in 6 different sets were set up (Suppl. Table A). P[w\'* mus301\']/TM3 progeny emerging from these vials were self-crossed. In the next generation, vials giving rise to >1/3rd of flies homozygous for P[w\'* mus301\']/P[w\'* mus301\'] are considered non-lethal events, while vials with either none or very few (≤5% of total population) homozygotes are considered lethal or semi-lethal events respectively.

“No donor” controls (2 lines): These control lines lack the P[w\'* mBF\’]. To establish these two lines, 3 iso males were crossed to I-Crel Sh/TM6 or I-Scel FLP/TM3 females separately. Vials were emptied after 3 days and the progeny larvae were heat shocked as mentioned above to produce the FLP recombinase.
Table 1. Results of homologous recombination

<table>
<thead>
<tr>
<th>Fly line</th>
<th>32A</th>
<th>32B</th>
<th>I-SceI</th>
<th>Stop</th>
<th>32A</th>
<th>32B</th>
<th>I-SceI</th>
<th>Stop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>-</td>
<td>WT</td>
</tr>
<tr>
<td>R1</td>
<td>M</td>
<td>WT</td>
<td>-</td>
<td>WT</td>
<td>M</td>
<td>M</td>
<td>-</td>
<td>WT</td>
</tr>
<tr>
<td>R2</td>
<td>M</td>
<td>M</td>
<td>-</td>
<td>M</td>
<td>M</td>
<td>WT</td>
<td>-</td>
<td>WT</td>
</tr>
<tr>
<td>R2*</td>
<td>M</td>
<td>M</td>
<td>-</td>
<td>M</td>
<td>M</td>
<td>WT</td>
<td>-</td>
<td>WT</td>
</tr>
<tr>
<td>R3</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>WT</td>
<td>M</td>
<td>M</td>
<td>-</td>
<td>WT</td>
</tr>
<tr>
<td>R4</td>
<td>WT</td>
<td>WT</td>
<td>-</td>
<td>WT</td>
<td>M</td>
<td>M</td>
<td>-</td>
<td>WT</td>
</tr>
</tbody>
</table>

32A: 32A ATG start codon; 32B: 32B ATG start codon; I-SceI: I-SceI site introduced into the intron between the unique 32B exon and the exon shared by 32A and 32B; Stop: conversion of the BamHI site in the shared exon into two tandem stop codons; R: Recombinant BEAF fly line; WT: wild-type BEAF sequences; M: mutated BEAF sequences; -: no I-SceI site. R2*: Fly stock derived from R2 by using I-CreI to reduce the gene duplication to a single copy. There is a mini-white gene between the upstream and downstream copies of BEAF. Reduction to a single copy also eliminated the mini-white gene. Gene-specific PCR was used to amplify the genes, followed by restriction digestion analysis and sequencing. The presence of M sequences where WT sequences were expected is unexpected. The presence of WT sequences where M sequences were expected is likely due to DNA repair occurring during branch migration during homologous recombination. These repair results are similar to those reported elsewhere.3,12,13

Table 2. Frequency of second site mutations

<table>
<thead>
<tr>
<th>Genotype of heat-shocked male larvae</th>
<th>No. of crosses</th>
<th>Lethal</th>
<th>% Lethal</th>
<th>Chi-square</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>w*; ada/B[hs-I-Cre1 Sb/</td>
<td>237</td>
<td>7</td>
<td>2.95%</td>
<td>0.0528</td>
<td>0.8183</td>
</tr>
<tr>
<td>w*; ada/B[hs-I-Cre1 Sb/</td>
<td>105</td>
<td>3</td>
<td>2.85%</td>
<td>0.0655</td>
<td>0.7980</td>
</tr>
<tr>
<td>w*; ada/B[70-FLP] [70I-Sce1]</td>
<td>108</td>
<td>4</td>
<td>3.7%</td>
<td>6.433</td>
<td>0.0112</td>
</tr>
<tr>
<td>P[w*mBf]; 3 iso/B[70-FLP] [70I-Sce1]</td>
<td>118</td>
<td>11</td>
<td>9.32%</td>
<td>3.913</td>
<td>0.0479</td>
</tr>
</tbody>
</table>

See the legend to Figure 3 for details.

References

and site-specific endonucleases.3,4 P[w*mus301/I-Cre1 Sb] or P[w*mus301/I-Sce1 FLP] males emerging from this cross were then individually mated with TM3/ET50 females. By this point ~150 vials for each control line were set up in 3 separate sets (Suppl. Table A). P[w*mus301]/TM3 adults eclosing (eliminating I-Cre1 Sb and I-Sce1 FLP chromosomes) were then self-crossed and in the next generation the number of lethal vs. non-lethal events were calculated.

Experimental lines (2 lines): First P[w*mBf]/P[w*mBf]; I-Cre1 Sb/TM6 and P[w*mBf]/P[w*mBf]; I-Sce1 FLP/TM3 stable lines were established. 3 iso males were crossed with females from each of the above two lines separately. Vials were emptied of flies after 3 days and they were heat shocked as mentioned above. Males of the P[w*mBf]; P[w*mus301/I-Cre1 Sb] or P[w*mus301/I-Sce1 FLP]; P[w*mus301/I-Sce1 FLP] were isolated and crossed to TM3/ET50 females. Around ~150 vials in 3 sets (Suppl. Table B) were set up by this point. In the next step males of the genotype P[w*mus301]/TM3 (getting rid of P[w*mBf], I-Sce1 FLP and I-Cre1 Sb) were crossed with TM3/ET50 females. Progeny males and females of P[w*mus301]/TM3 genotype were then self-crossed. Progeny from these crosses were scored to determine the number of lethal vs. non-lethal events.

Acknowledgements
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Note
Supplementary materials can be found at:
www.landesbioscience.com/supplement/RoyFLY4-1-Sup.pdf


