Gene Targeting of a Plasmid-Borne Sequence to a Double-Strand DNA Break in Drosophila melanogaster

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We report an efficient and specific gene targeting method for transforming the germ line of Drosophila melanogaster. The targeting occurs during the repair of a double-strand DNA break that is induced at the white locus by the excision of a P transposable element. The break is repaired when homologous sequence is copied from a plasmid injected into the Drosophila embryo. The procedure efficiently integrates DNA into the targeted locus of the Drosophila genome. Heterologous sequence of up to 13 kb in length can be inserted, permitting the integration of entire genes into a common genomic site for further study.

Gene targeting is used to insert or alter a defined genomic sequence in a living cell. The current method is especially useful with single-celled eukaryotes such as yeasts and trypanosomes (4, 20, 27). However, it is less useful in multicellular eukaryotes such as Drosophila melanogaster, Caenorhabditis elegans, and mammals because of the relatively low efficiency and specificity in recovering a defined genomic insertion (15). An efficient and specific gene targeting system is required to make alterations in the larger and more complex metazoan genomes.

Gene targeting in D. melanogaster can be performed in several ways. One method, which is the same as that used in single-celled eukaryotes and mammalian systems, involves transfection of linear DNA into tissue culture cells and integration by homologous recombination (1a). This method generally suffers from a lack of specificity and currently can be used only to transform Drosophila tissue culture cell lines. However, in theory every genomic site can be targeted.

We and our collaborators have devised a novel and efficient gene targeting technique based on the repair of a double-strand DNA break made in a chromosome (6, 8, 17). The method permits the introduction of DNA sequences into the region of a preexisting transposable element insertion. Gene targeting in this system is initiated by excision of the P element (19) from the X-linked w yellow allele (6). Excision of the P element results in a double-strand DNA break at the white locus (6, 8, 14). This break is repaired when DNA sequence is copied from a homologous template sequence (6, 8). The repair process resembles the gene conversion events that occur during the repair of a double-strand chromosome break in yeast cells except that in Drosophila cells, there is little or no associated crossing-over of flankng genetic markers (6, 8). This approach can be used to target homologous sequences, sequence deletions, or heterologous sequences to the region of the double-strand break (17). A related phenomenon can be used to insert heterologous sequence at a specific site by replacement of the excised P element with a different P element (7, 10, 11). Both of these techniques are very efficient and specific but rely on the presence of a preexisting P element insertion near the targeting site.

Previous experiments demonstrated that a homologous DNA sequence can be copied into the break site from individual templates inserted at 3′-end in the genome (5, 8, 17). These observations suggested that a genome-wide search for sequences homologous to those flanking the break could be initiated. In this report, we introduced the homologous sequence on a plasmid to test if homologous and heterologous sequences on nonintegrated templates could also be copied into the break site.

MATERIALS AND METHODS

Genetic screen for reversions. Genetic symbols not defined here are as defined by Landsley and Zimm (16). Drosophila stocks were maintained on a standard cornmeal-sucrose-yeast-agar formulation. Crosses were performed at room temperature 24°C unless noted otherwise, brooded at 5 to 7 days, and scored until 1 to 2 days before the F2 generation was expected to eclose (about 18 days at 24°C). Drosophila embryos were injected through the chorion as described previously (22). The template plasmids and transposase-making helper plasmids were injected at approximately 1 and 0.25 mg/ml unless noted otherwise. The scheme for recovering template-dependent reversions of w yellow is shown in Fig. 1. The P element excision and gap repair events occur in the germ line of the parental male, which carries an immobile P element transposase source, /+;3(9B)B (22), the w yellow target site, and one of the injected template constructs described in Fig. 2. Revertant progeny were identified by their wild-type eye color.

The white gene-containing templates are named according to the presence or absence of a heterologous insertion (Fig. 2) and according to the presence or absence of P-element ends. Templates cloned in the pBluescript II vector (Stratagene) are termed pBS vectors or nontransposable vectors, and templates cloned between two P-element ends are termed pP[white(etc.)] or transposable vectors.

Molecular analyses of revertants. DNA for PCR was prepared from individual flies as described elsewhere (9). Primers and their sequences are described below. Each DNA sample was amplified with primer pairs p1-p2 and p3-p4 to determine if the left and right P-element ends of the template were present. Flies positive for both P ends were presumed to carry a copy of the template transposon and were discarded without further analysis. The rest were analyzed by PCR and restriction mapping as described below to determine the structure of the revertant allele and the extent of the conversion tract. Additional PCR amplifications were used to determine the structure of more complex events.

Oligonucleotide primers for PCR analysis of the reversions. PCRs to test for linkage of the white gene and the P-element ends were done with the oligonucleotide primers pairs p1-p2 for amplification from the P 3′ end to the white 3′ end and p3-p4 for amplification from the P 3′ end to the white 5′ end. Flies giving amplification products were scored as positive for linkage. The primer pairs used to analyze the site(s) shown in Fig. 3 were p5-p6 (site 1), p7-p8 (site 2), p9-p10 (site 3), p11-p12 (sites 4, 5, 6, 7, 8, 9, and MCS [multiple cloning site]); p13-p14 (sites MCSs and 9); and p15-p16 (sites 9n and 10). When the insertion was copied in wAl vectors, the sizes of the amplified products from p11-p12 and p13-p14 were 25 bp larger. For the wAl reversions, we used p11-p17 (yellow gene 5′ end) and p14-p18 (yellow gene 3′ end). The 3′ ends of the duplications were analyzed by amplification with p11-p19, from which we could analyze sites.

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RESULTS

These experiments were designed to test if homologous sequences could be copied from nonintegrated templates into the white locus. The method used to recover template-dependent reversions of the \(w^{alL}\) allele is shown in Fig. 1. In this experiment, a double-strand DNA break is induced at the Drosophila white locus by excision of the \(P-w^{alL}\) element. This is repaired by copying sequence from a homologous template injected into the posterior region of syncytial blastoderm embryos where the pole cells will develop. The injected templates all carry a mini-white gene containing multiple single-base alterations from the canonical white gene sequence (8, 17) so that progeny containing an integrated white gene can be identified. Male flies that survived the injection process were mated, and their progeny were examined for their eye phenotype. Those with either orange or red eyes were examined further for evidence that DNA from the injected template had been copied into the white locus.

Conversion from injected plasmids. In the first experiment, a template plasmid (pP[walter] [Fig. 2]) and the transposase source (p\(\pi\)25.7wc [13]) were coinjected into embryos with the genotype \(y \text{, } w^{alL}\). We reasoned that conversion of the injected DNA into the white locus would occur if transposase excised the \(P-w^{alL}\) element in cells that contained the template plasmid. In this experiment, the modified white gene template (walter) was located between two P-element ends. The resulting construct, pP[walter], either could serve as a repair template or could transpose the white gene into a random genomic site in the Drosophila genome (24, 26). Plasmids like pP[walter] that have the white gene flanked by P-element ends will be referred to as transposable templates. Random integration of these templates by transposition into the Drosophila genome would be described previously (9). Standard dideoxy sequencing procedures were then applied (25).

FIG. 1. Mating scheme for gap repair. Drosophila embryos with the genotype \(\text{C(1)DX,w}^{\text{alL}}\) were injected with the template plasmid at a concentration of 1 mg/ml. The transposase source caused \(P-w^{alL}\) to excise. Embryos were grown to adulthood and mated to a compound-X stock, and the eye phenotype of each progeny male was examined. Those retaining the parental white eye color were not analyzed further. Red-eyed progeny of the male was examined. Those retaining the parental white eye color were not analyzed further. Red-eyed progeny of the male was examined. Those retaining the parental white eye color were not analyzed further. Red-eyed progeny of the male was examined. Those retaining the parental white eye color were not analyzed further. Red-eyed progeny of the male was examined. Those retaining the parental white eye color were not analyzed further. Red-eyed progeny of the male was examined. 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serve as a positive control for transposase activity. The results (Table 1, experiment 1) show that both types of events were recovered. The red-eyed flies from this and subsequent experiments were analyzed for the presence of DNA sequence copied from the injected template as shown in Fig. 3. The analysis demonstrated that >90% of the red-eyed male flies recovered in this and subsequent experiments were convertants.

In the second experiment, the same template was injected into embryos containing a stable genomic transposase source, D2-3(99B) (22). In this experiment, excision of the $P_{\text{whd}}$ element could occur in any cell throughout the flies life cycle and not necessarily in cells that had received the injected template. The results of experiment 2 (Table 1) show that both red-eyed convertants of $w^{\text{nd}}$ and orange-eyed transformants were recovered.

**Conversion is independent of template $P$ ends.** Previous experiments in which the template was located ectopically were always performed with templates containing at least one $P$-element end (5, 8, 17). It was possible that the presence of $P$-element ends on the template contributed to the repair process. We therefore wanted to determine if the template white gene sequence was copied directly from the injected template or was copied from a randomly integrated $P[\text{walter}]$ element. These possibilities were tested in an experiment in which the templates were cloned into the pBS vector and lacked $P$-element ends. These templates would therefore not be expected to transpose the white gene into random genomic sites.

There were 73 fertile male flies resulting from these injections that used either a co-injected (Table 1, experiment 3) or genomic (Table 1, experiments 4 and 5) transposase source. These males produced 5,498 progeny, of which 35 were red-eyed and had copied sequence from the templates into the white locus. No orange-eyed progeny were recovered. This result ruled out the possibility that the plasmid had integrated into the genome. We concluded that the conversions resulted from DNA sequence being copied directly from the injected plasmids and not from integrated templates.

**Concentration dependence of conversion.** The next experiment tested how the concentration of the injected template affected the efficiency of conversion. The genomic transposase source was used exclusively for these experiments, since the data in Table 1 (experiments 3 to 5) indicated that conversion was more efficient with it than with the co-injected helper transposase source. Four different template concentrations, ranging from 250 µg/ml to 2 mg/ml, were used. The results of this experiment are shown in Table 2. We concluded that the op-

<table>
<thead>
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<th>Expt</th>
<th>Template</th>
<th>Transposase</th>
<th>No. of:</th>
<th>Fertile males</th>
<th>Progeny</th>
<th>Transformed$^a$</th>
<th>Convertants$^b$</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>pP[walter] Plasmid</td>
<td>19$^c$</td>
<td>845</td>
<td>1 (2)$^d$</td>
<td>1 (15)$^e$</td>
<td></td>
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<tr>
<td>2</td>
<td>pP[walter] Genomic</td>
<td>7$^c$</td>
<td>195</td>
<td>5 (49)$^d$</td>
<td>1 (15)$^e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>pBSwalter Plasmid</td>
<td>37$^c$</td>
<td>2,229</td>
<td>0$^c$</td>
<td>1 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>pBSwalL Genomic</td>
<td>24$^c$</td>
<td>2,299</td>
<td>0$^c$</td>
<td>6 (9)$^e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>pBSwalLy Genomic</td>
<td>12$^c$</td>
<td>970</td>
<td>0$^c$</td>
<td>6 (21)$^e$</td>
<td></td>
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</tr>
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</table>

$^a$ Number of males transmitting transformants (total number of transformants).

$^b$ Number of males transmitting convertants (total number of convertants).

$^c$ One red-eyed fly was not characterized.

$^d$ Represents approximately 2.5% of injected flies (injections done at 27°C).

$^e$ The white gene could not integrate by transposition.

$^f$ Includes at least one sibship that segregated different conversion tracts.

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**FIG. 3.** Simple conversion tracts. DNA prepared from single flies was analyzed by using PCR to amplify regions of the white gene. The amplified segments were restriction digested to determine if they corresponded to template or normal white gene sequence. The target symbol represents a variable amount of heterologous sequence as shown in Fig. 2. The relative position of each altered site with respect to the chromosomal break is given above the numbered sites. Asterisks denote the most extreme case of four different conversion tracts recovered from sibling flies. §, the partially copied yellow gene.
timum template concentration was about 1 mg/ml with our injection technique.

**Conversion frequencies.** We estimated the frequency of conversion from the injected templates by pooling the data for those experiments that used both the genomic transposase source and transposable templates at similar concentrations (Table 1, experiment 2; Table 2, 1,100 μg/ml). With these templates, about 4.7% of the progeny were transfectants (177 transfectants from 3,805 progeny), and about 1.4% of the progeny were revertants (177 revertants from 3,805 progeny), and about 1.4% of the progeny were revertants (177 revertants from 3,805 progeny). Apermation test was used to compare these frequencies, and we concluded that the frequencies of transformation and conversion were significantly different (P < 0.0001).

We wanted to assess the contribution of the P-element ends to the conversion process by comparing the conversion frequencies for the transposable and nontransposable templates. This comparison is difficult since, unlike the comparison between transposition and conversion described above, it is not within one data set. Nevertheless, the pooled data for the nontransposable templates (Table 1, experiments 4 and 5) show that 36 injected flies had 3,269 total progeny with at least 12 independent transfectants (30 total transfectants). This compares favorably with the transposable templates described above, in which case 70 injected flies had 3,805 total progeny with at least 5 independent transfectants (54 total transfectants). We suggest that the template P-element ends play little or no role in the conversion process from a plasmid template.

**Simple conversion tracts.** Figure 3 shows the amount of sequence copied from the injected plasmids into the white locus of each revertant. Twenty-one males produced revertant progeny (Tables 1 and 2). However, 25 different conversion tracts were observed. The progeny of a single injected male often included more than one revertant, and some siblings segregated more than one conversion tract. In the extreme case, one male, injected with pBSwalL, had 11 revertant progeny that segregated four different conversion tracts. These included some that converted the yellow gene and others that did not (these four conversions are noted with asterisks in Fig. 3 and 4). Different conversion tracts recovered from a single injected male are consistent with independent events occurring in more than one pole cell in an injected embryo.

Eighteen of 25 conversion tracts copied a continuous block of template sequence into the white locus without deletion or duplication formation. All of the conversion tracts copied the central base alteration located at the site on the template that corresponds to the break site in the white locus. Conversion of the flanking sites decreased with increasing distance from the central site as was observed previously (8, 17).

**Duplicated conversion tracts.** Almost half (6 of 13) of the conversion tracts copied from the nontransposable templates were tandem duplications of the white gene with the pBS vector sequences between them (Fig. 4). The structure of each of these duplications was confirmed in three ways. In the first experiment, a simple conversion and a duplicated conversion were tested by in situ hybridization with a white gene probe. The probe was observed to hybridize only at the white gene’s proper cytological position in both (21a). In the second experiment, PCR amplification was performed with primers p2 and p20 and with primers p3 and p21. These primer pairs specifically amplify the white-pBS junctions. This PCR experiment confirmed the linkage of the white gene and the pBS vector in the genomic DNA (data not shown). In the third experiment, the pBS vector and associated white gene sequence were rescued from the genomic DNA. Three micrograms of genomic DNA was isolated from three of the duplications and digested with HindIII or with KpnI. These restriction endonucleases recognize a unique site on one side or the other of the integrated plasmid (Fig. 4). The DNA was circularized by ligation and transformed into E. coli. We recovered white genes with the appropriate conversion tracts as plasmids (data not shown).

One trivial explanation for the origin of the duplicated insertions is that they could have been copied from plasmid dimer templates. This was tested in two ways. First, 1 μg of each of the pBS plasmid preparations was electrophoresed on an agarose gel. Ethidium bromide staining revealed no plasmid dimers (data not shown). In a second experiment, five independent larvae that had been injected with pBSwalL were recovered at 48 h postinjection, washed, and macerated. The mixture was centrifuged, and the supernatant was used to transform E. coli cells. The template plasmids from 97 independent colonies were isolated and analyzed by agarose gel electrophoresis. All of the plasmids recovered were monomers (data not shown). We concluded that less than 1% of the injected template DNA was in the form of plasmid dimers. This amount of plasmid dimer was inconsistent with the high frequency of duplicated conversion tracts recovered from these templates.

A mixing experiment was performed to determine how the duplicated structures were formed. The pBSwalL and pBSwalter templates were mixed at a 1:1 ratio and coinjected. These templates differed at two sites: sites 3n and PL (Fig. 3) were present in pBSwalL and absent in pBSwalter. Therefore, the sites present on each side of the pBS vector in the duplicated conversion tracts would represent their origin. Four informative conversion tracts were recovered in this experiment. Three of these were duplicated and had conversion tracts extending beyond sites 3n and PL (Fig. 4). In each, sequence on both sides of the duplication was derived exclusively from pBSwalL.

Duplicated conversion tracts were observed only with the nontransposable templates. The progeny derived from males injected with transposable templates were examined for the presence of both P-element ends in an initial PCR screen. This preliminary analysis ensured that conversion tracts for random transformants were not determined. However, the analysis also prevented the recovery of duplicated convertants copied from the transposable templates.

**Conversion of heterologous insertions.** Heterologous sequences could also be copied from the template into the white locus (Fig. 3 and 4). When the templates included a nonhomologous DNA insertion, it was copied into the break site.
almost 50% of the time (7 of 16 instances) (Fig. 3). Such insertions were included in 51 to 68% of the conversion contracts in previous experiments (17). The results shown here indicate that conversion of heterologous sequences is approximately as efficient from plasmid templates as from ectopically inserted genomic templates.

Two of the heterologous sequences, the forked and yellow genes, produced a visible phenotypic change when they were converted. In these experiments, one half of the conversions copied from the injected p[wal] template were forked+, indicating that the conversion tract included the entire heterologous insertion. The forked gene is about 5 kbp larger than the 8 kbp yellow gene and is the largest heterologous sequence that has been copied into the white locus to date (17). Therefore, conversion of the yellow and the forked genes demonstrates the utility of this procedure for the targeted transformation of long sequences into a defined genomic site.

Almost all (24 of 25) of the conversion tracts precisely copied template sequence into the break site. We noted that one of the conversions copied one end of the yellow gene from pBSwalL but not the other. Such events have been observed previously and may be the product of interrupted copying as described elsewhere (17).

**DISCUSSION**

One of the major problems encountered when a DNA sequence is introduced into a cell is the variation in gene expression caused by random insertion (15, 29). This variability places severe constraints on interpretation of the experiments and on the types of experiments that can be performed. One solution to these position effects would be to place all of the constructs at a common genomic site where the position effects on expression are nonexistent or at the very least are reproducible. Such an approach is justifiable only if the integrated sequences can be recovered relatively easily. Current methods for gene targeting result in a relatively low rate of targeted insertion (15).

We have described an efficient and specific method for targeting DNA sequences into the *Drosophila* white locus by copying them from an injected plasmid. We observed that both point mutations and heterologous DNA sequences up to 13 kbp long could be copied from the template into the break site. The nonhomologous DNA was copied about as efficiently as a single-point mutant, as has been observed for templates located both on the homolog and ectopically (17). As a result, the integrated gene will be subject to a predictable position effect. The ability to use plasmid templates to insert heterologous sequences into the genome provides a distinct advantage over a previous method that used oligonucleotide templates for gap repair (1).

**Optimization of conversion.** The efficiency of conversion was optimized in three ways. First, conversion events were recovered more frequently when transposase was supplied from the genomic source than when it was supplied from a co-injected helper plasmid. This may be because the double-strand break exists before injection of the template DNA or because more transposase is made from the genomic transposase source. Second, conversion was most frequent when the template was injected at a concentration of about 1 mg/ml. Finally, the role of *P*-element ends on the template was investigated. The results indicated that conversion from an ectopic template was unaffected by template *P*-element ends.

**FIG. 4.** Duplicated conversion tracts. The symbols are as for Fig. 3. These conversion tracts had two copies of the white gene separated by one copy of the template vector sequence. The asterisk denotes the most extreme case of four different conversion tracts recovered from sibling flies.
Further optimization should be possible. For example, a heterologous sequence would be copied more frequently if it was inserted in the template at the site corresponding to the chromosomal break site (12). Additionally, these repair templates had 15 single-base differences from the genomic white gene sequence. It is likely that the targeting efficiency would increase severalfold if the template sequence was identical to the break site except for the mutation or insertion to be introduced (3, 18, 28).

One additional parameter that is likely to influence the process is the P-element excision rate. The P-w + element is very mobile; it transposes to an average of 1.5 new genomic sites per generation (6). Therefore, conversion at other loci is expected to occur at a frequency that is different from the conversion frequency at the white locus. We suggest that any difference would be largely dependent on the excision rate of the resident P element.

This transformation method should be applicable to other P-element insertion alleles. In support of this view, plasmid templates have been successfully used for gene targeting at the hoxc locus (20a).

Conversion frequencies. In the experiments conducted with transposable and nontransposable template concentrations of about 1 mg/ml, the pooled average conversion frequency was 1.2% (84 convertants from 7,074 progeny). This frequency is about fourfold lower than the frequency of random integration that was observed (4.7%). Nevertheless, the conversion frequency is high enough that PCR-based screens for convertants would be relatively simple. Such a screen has been successfully performed for conversion at the white locus by another group (5a).

It is noteworthy that the conversion frequency observed in this experiment was similar to the average conversion frequency when the templates were inserted into random sites on the Drosophila autosomes (8, 17) or were located on the X chromosome in trans to the break (5). However, the conversion frequency from plasmid templates is significantly lower than from X-linked templates located in cis to the break (5). This finding suggests that injected plasmid templates are found during the homology search about as efficiently as autosomally inserted templates or X-linked templates located in trans to the break.

The experiments reported here are similar to two recently reported experiments in which defined chromosome breaks were introduced into the genome of mouse cells grown in culture (2, 23). In these experiments, the double-strand breaks were introduced by cleavage with the rare-cutting endonuclease I-ScI. One group observed that up to 10% of the cells transfected with the endonuclease incorporated sequence from a cotransfected homologous template (23). It is interesting to speculate that better control of the strand breakage step in the Drosophila system would result in a corresponding increase in conversion frequency.

Duplication formation. Almost half (6 of 13) of the conversions copied from the nontransposable templates were tandem duplications. The data indicated that the duplications did not occur by conversion from templates that had duplicated either before or after injection into the embryos.

Three possible ways that duplications could form from monomer templates are shown in Fig. 5. The first possibility, shown in model A, is that the duplications were formed by a homologous recombination event occurring during gap repair. This possibility is unlikely since significant recombination rates are not observed with genomic templates inserted either ectopically or on the homologue (6, 8, 17). The second possibility, shown in model B, is that the dimers formed when the two broken ends invaded and copied sequence from different plasmid templates. The two copied sequences would base pair at homologous sequences to complete the chromosome repair. This mechanism is consistent with a previously described model that was used to explain how a duplication might form when an ectopic template is used (17). However, this mechanism would be expected to give duplications in which each side had sequence derived from a different plasmid template in a mixing experiment. This was not observed. Finally, the duplications could arise by DNA replication proceeding twice around the inverted template as shown in model C. We favor this mechanism for two reasons. First, the mixing experiment showed that three independent duplications were derived from a single template. Second, duplications were much more frequent when the template contained a 25-bp heterologous insertion (pBSwaiI; five of seven instances) than with an otherwise identical template that contained an 8-kbp heterologous sequence (pBSwaiII; one of six instances). Such an insertion would significantly increase the time required for replication to proceed twice around the template and thus would be expected to reduce the formation of these structures.

Transformation by conversion into double-strand DNA breaks should be applicable to other organisms in which these breaks can be made. Similar systems have been developed in mouse cells by using rare-cutting endonucleases to produce the double-strand DNA break (2, 23). It appears that double-strand break repair will prove useful for targeted gene insertion in many metazoan eukaryotic systems in which specific double-strand DNA breaks can be made.

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REFERENCES


2. Cherbas, L., and P. Cherbas. Personal communication.


