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FLP-mediated DNA mobilization to specific target sites in *Drosophila* chromosomes

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ABSTRACT

The ability to place a series of gene constructs at a specific site in the genome opens new possibilities for the experimental examination of gene expression and chromosomal position effects. We report that the FLP–*FRT* site-specific recombination system of the yeast 2μ plasmid can be used to integrate DNA at a chromosomal *FRT* target site in *Drosophila*. The technique we used was to first integrate an *FRT*-flanked gene by standard *P* element-mediated transformation. FLP was then used to excise the *FRT*-flanked donor DNA and screen for FLP-mediated re-integration at an *FRT* target at a different chromosome location. Such events were recovered from up to 5% of the crosses used to screen for mobilization and are easily detectable by altered linkage of a *white* reporter gene or by the generation of a *white*$^+$ gene upon integration.

INTRODUCTION

Current methods for transformation of *Drosophila* utilize transposable elements as vectors to carry DNA into the genome. Because these elements transpose into essentially random locations, transformed genes end up scattered throughout the genome. Their expression is subject to the influences of their immediate chromosomal environments. These position effects on gene expression are quite commonly observed and in some experiments are a serious nuisance. For instance, if a series of genetically engineered gene alterations are to be compared, position effects on the integrated constructs complicate the interpretation of results. The typical solution is to assay a number of independently derived transformants of each construct and average the results (1). If a single transformant of each construct could be analyzed the amount of work could be significantly reduced. The degree of confidence in such averaged results is also diminished, because a single extreme position effect can strongly influence the average.

One solution to this problem is to target the integration of all constructs to a single site, where they would all be subject to the same position effect. Conclusions derived from such comparisons are more compelling because a major source of variability is eliminated. This can be achieved in yeast and mice by using homologous recombination to place DNA at a specific site (2,3). In plants, where homologous recombination techniques have not been developed, site-specific recombination has been used to integrate DNAs at a target site for a recombinase that was previously integrated into the genome (4).

DNA placement by homologous recombination and by site-specific recombination have so far only proven useful in systems where chemical selection can be applied to single cells. With both techniques integration events are relatively rare (typically 10^{-4}–10^{-6}). *Drosophila* does not readily lend itself to the type of chemical selection that would be required to recover such rare events. No method of cell culture has been reported that would allow chemical selection on isolated cells with subsequent return of the selected cells to the germline of an intact animal. Transformants are obtained in *Drosophila* by injecting DNA into very young embryos, mating the adults that survive this procedure and screening their progeny for flies that carry the DNA that must have integrated into the germline cells of their injected parents (5). Although chemical selection has been used to recover transformants among the progeny of injected flies, the technique does not greatly increase the number of progeny that can be screened. In contrast to cell culture systems, in which selection can be applied to millions of cells in a single Petri dish, embryo injection is still required as the first step when chemical selection is utilized in *Drosophila*.

We set out to use site-specific recombination to target DNA integration to specific sites in the *Drosophila* genome. Because of the difficulties described above, it seemed that using site-specific recombination to effect the direct integration of injected DNAs would not be feasible as a first step. Instead, we chose to utilize a method that would allow us to place a copy of the DNA that we wished to integrate (the donor) into every germline cell of whole animals. We used standard *P* element transformation to integrate the donor DNA in the *Drosophila* genome. This *P* element carried direct repeats of the target site (*FRT*) for the FLP site-specific recombinase (6) flanking the donor DNA. FLP can excise the *FRT*-flanked DNA from the chromosome with nearly 100% efficiency (7,8) and the DNA is excised as an intact circular DNA

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that carries one *FRT* (9). Therefore, FLP-mediated excision can generate a single episomal copy of the donor DNA in virtually every cell in an animal. If such an animal also has an *FRT* integrated at another site (the target), then when the extrachromosomal donor and the chromosomal target come into contact, FLP can mediate a second round of recombination that will integrate the donor DNA at the target site. The method is conceptually simple and abundant evidence demonstrates that site-specific recombination can be used to effect this DNA integration in other organisms (10–13). The problem has been to find experimental conditions that take into account the biology of *Drosophila* so that the targeted integration event will occur with sufficient frequency. We describe experimental parameters that can be used to recover targeted integrants using the FLP site-specific recombinase in *Drosophila melanogaster.*

MATERIALS AND METHODS

All *P* element constructs were transformed by standard techniques (5). Heat shocks were performed in a circulating water bath as previously described (7).

FLP and *FRT* constructs

The *hsFLP* gene has been previously described (7).

A second source of FLP used in these experiments was an *hsp70–FLP* fusion gene called *70FLP.* It is carried in a *P* element marked with ry^+, called *P*[ry^+, *70FLP*]. It was constructed by cloning the *Xba*I–*Sal*I *FLP* fragment from pDM420-FLP (7) into the plasmid pVZ1. This was transformed into strain RZ1032 (dut^-/ung^-; provided by S.Henikoff, Fred Hutchinson Cancer Research Center). Single-stranded DNA was prepared from a 20 ml culture and mutagenized to add a *Xho*I site at the 3′-end of the gene by synthesizing the second strand using a phosphorylated primer containing the mutation. The primer sequence was 5′-GCTTAAATGCTCGAGCTTATATGCG-3′. The resulting plasmid was called pFLP-XhoI. The mutated *FLP* gene was cloned into the plasmid p70ATG-Bam in which the normal *hsp70* translation start site was replaced by a *Bam*HI site (14). This was accomplished by cutting p70ATG-Bam with *Bam*HI and filling in the 5′-overhang and then cutting with *Sal*I to remove the *hsp70* sequences. The *FLP* coding sequence was obtained by cutting pFLP-XhoI with *Sal*I and filling in the 5′-overhang and then cutting with *Xho*I to isolate the *FLP* sequence from the vector, which was then ligated into the prepared p70ATG-Bam vector. The resulting plasmid was called p70/Flp/70. To facilitate moving the *hsp70–FLP* fusion gene into a *P* element vector the partially cleaved fusion gene (*Eco*RI and *Hin*dIII) was cloned into *Eco*RI and *Hin*dIII-cleaved pHSS6 (15), which served as a shuttle vector by providing flanking *Not*I sites. After isolation of the *Not*I fragment from the shuttle vector it was cloned into *Not*I-digested pDM30 (1), yielding the plasmid p*P*[ry^+, *70CFLP70*], or simply *70FLP. 70FLP*3F is an insertion of this gene on the X chromosome.

A third *FLP* gene used here is the β*2t–FLP* gene. This was constructed by cloning the β*2tubulin* promoter from pUMB2 (16) into pHSS6 as a 0.7 kb *Eco*RI–*Hin*dIII fragment. The coding sequence of *FLP* was amplified by PCR with primers 5′-GGGATCCAAGCTTGCGCAGCTGAACAAGCTAAAC-3′ and 5′-GTCGACTCTAGAGCGCTTCCGAAAATGCAAC-3′, using a plasmid carrying *hsFLP* as template. These add *Hin*dIII and *Xba*I sites to the *FLP* coding sequence and these sites were used to insert the coding sequence behind the β*2t* promoter in pHSS6. The β*2t–FLP* gene was removed as a *Not*I fragment and cloned into the *Not*I site of the *P* element vector pDm30.

Two donor constructs were used in this work. The *P*[>w^{hs}>] element has been described previously (7). *P*[*X97*] was constructed by placing a *Spe*I linker with stops in all three reading frames (NEB 1061) into the *Pvu*II site in the second exon of w^{hs} in plasmid *P*[*X96*] (9). This plasmid has a unique *Not*I site between the two halves of the w^{hs} gene. An *hsGFP* fusion gene (unpublished) was placed into this *Not*I site to generate the *P*[*X97*, *hsGFP*] element that was used in these experiments.

Two *FRT*-containing target site constructs were used: *RS5r* and *RS3r.* Their construction has been described (17).

PCR and primers

To confirm that the *P*[*X97*]-derived donor DNA was integrated at the *RS3r* target sites as expected we used genomic DNA from the putative integrants as template for PCR. The primer pair used [5′-GATAGCCGAAGCTTACCGAAGT-3′ and 5′-TCATCG-CAGATCAGAAGCGG-3′] amplifies fragments of characteristic size from *P*[*X97*, *hsGFP*], *RS3r* and from the donor DNA integrated at *RS3r.* The sizes and locations of these fragments are indicated in Figure 2.

RESULTS

The $white^+$ gene construct used in these experiments is actually a mini-*white* gene (18) that we call w^{hs}. All flies used in these experiments carried the w^{1118} null mutation. In a w^{1118} background w^{hs} usually produces an orange colored eye; for the sake of simplicity we refer to this phenotype as $white^+$ (w^+).

Detecting germline mobilization by altered genetic linkage

Our initial attempt to recover site-specific integrants used a scheme to detect FLP-mediated mobilization of an *FRT*-flanked *white* gene from a site on the X chromosome to an autosomal target *FRT* (Fig. 1). By inducing FLP synthesis in males that carried the donor and the target and mating those males to w^{1118} females we could easily detect mobilization to the target site. Normally the sons of this cross receive the w^{1118} chromosome from their mothers and have white eyes. If the *FRT*-flanked w^{hs} gene was moved to the autosomal *FRT* target site in the father's germline, a son with pigmented eyes (w^+) could be produced. In several combinations we recovered fertile sons with pigmented eyes (Table 1). From 1107 tested males we recovered 13 fertile w^+ males. We also recovered 17 sterile w^+ males, which most likely arose by non-disjunction of X chromosomes in the female parent to produce XO sons that were w^+ because they carried their father's X chromosome.

We used several criteria to determine whether the fertile w^+ sons carried the expected FLP-mediated integrant. First, the $white^+$ function was mapped to determine whether it was located on the target chromosome. In all 13 cases it was. Second, we tested whether the integrated gene was capable of re-excision by FLP. If it inserted at the target *FRT* by FLP-mediated recombination it should re-excise with FLP. By crossing to *hsFLP*-bearing flies, heat shocking the progeny early in development and then looking for somatic mosaicism we found that in 10 of 13 cases the

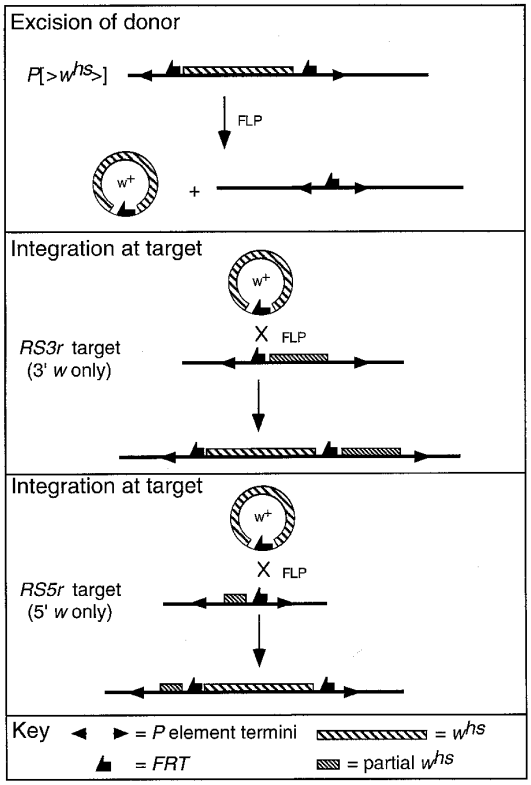

Figure 1. The method of FLP-mediated DNA mobilization. An *FRT*-flanked w^{hs} gene is first excised by FLP from one chromosomal site. Subsequent re-integration at a target site on another chromosome is detected by a screen for altered linkage, as described in the text.

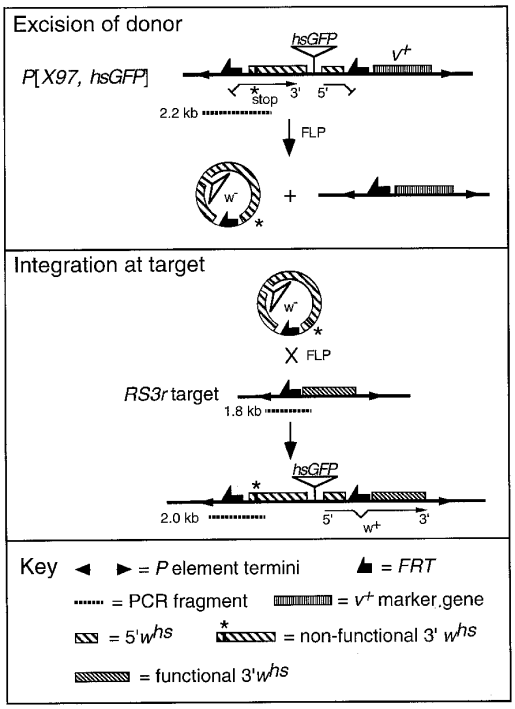

Figure 2. The method of mobilization using *P*[*X97*]. The *P*[*X97*] vector carries the w^{hs} gene split into two halves (as indicated) at a site in the first intron. (The remaining introns are not indicated.) The 3′ portion of the gene is disrupted by insertion of a stop codon. Passenger DNA (in this case the *hsGFP* gene) can be inserted at a unique *Not*I site between the two halves of the w^{hs} gene. FLP-mediated excision followed by re-integration at an *RS3r* target site regenerates a functional w^{hs} gene as diagramed.

gene could be re-excised by FLP, but in three it could not. An additional test confirmed that the first 10 were FLP-mediated integrants at the target site. Since the target *FRT* was carried within a *P* element, the integrated *white* gene should be mobilized by *P* transposase. We assayed each integrant for somatic mosaicism caused by expression of transposase from the transposase source Δ2-3(99B) (19). The 10 integrants that were excised by FLP were also mobilized by transposase; the remaining three were not. Finally, nine of the integrants that could be re-excised with FLP were tested by genomic Southern blotting and all had the expected bands. The three cases that did not produce mosaicism with FLP or transposase did not exhibit the bands expected for integration at the target *FRT* (data not shown). We conclude that 10 of the 13 recovered autosomal insertions are true examples of targeted mobilization by FLP, for an overall rate of 0.9%. (Throughout this paper, recovery is reported as the number of independent targeted integrants divided by the total number of vials screened.)

The scheme we used in these first experiments required donor insertions on the X chromosome. Autosomal donors could also be used if the chromosomes were appropriately marked. As an example we recombined a donor *P*[>w^{hs}>] element onto chromosome 3 marked with the dominant mutation *Diachete* (*D*). We then screened for FLP-mediated mobilization to target sites on chromosomes 2 or 3 by looking for w^+ D^+ progeny. From 406 vials we recovered four w^+ D^+ progeny (Table 2). All four appeared to be genuine FLP-mediated integration events, as judged by re-excision with FLP. Thus FLP can be used to mobilize genes from the X chromosome or autosomes to autosomal target sites.

Table 1. X Chromosome to autosome mobilization of a *white* gene

Donor	Target				
	RS3r-2(75C-D)	*RS3r*-3(82C)	*RS5r*-2B(II)	*RS5r*-1A(65B)	*RS5r*-2A(88B)
P[>w^{hs}>]	2/233	6/216	0/64	0/92	2/502[a]
(two copies on X)	0.9%	2.8%	–	–	0.4%

w^{1118} *P*[>w^{hs}>] *P*[>w^{hs}>]; *hsFLP*2B/+ males, that also carried a single copy of the indicated target site element, were generated by crossing and heat shocked at 37 or 38°C for 1 h during the first few days of development. The adults were mated to w^{1118} females (typically as one male with two females) and their progeny screened for w^+ sons. Results are reported as (vials with fertile w^+ sons)/(total vials). The cytological locations or linkage group are indicated in parentheses.

[a]Three additional independent fertile w^+ sons were recovered from this combination, but were not cases of FLP-mediated integration at the target *FRT* (see text).

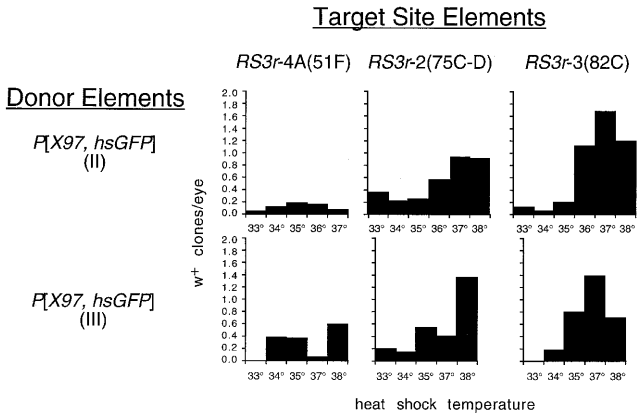

Figure 3. Targeted mobilization efficiency in the soma. Two *P*[*X97*, *hsGFP*] insertions were mobilized, by heat shock induction of *70FLP*, to three different *RS3r* target sites (see text for details). When targeted mobilization occurs in the developing eye, it produces w^+ clones that are easily scored. This figure shows the variation in clone frequency with varying heat shock temperatures. An average of 41 eyes were scored for each combination of donor, target and temperature.

Optimizing FLP induction

It is likely that efficient mobilization requires an optimal concentration of FLP: enough to catalyze excision and re-integration, but not so much that re-excision inevitably follows (13). The severity of the heat shock used to induce FLP synthesis strongly influences the quantity of FLP that is made (7). We used a somatic assay to determine the best heat shock conditions for obtaining site-specific integration. For this test we used the donor construct *P*[*X97*]. This construct carries an intact 5′-portion of w^{hs}, but the 3′-portion has been mutated by insertion of a stop codon in the second exon, so that unequal sister chromatid exchange cannot produce a functional gene. The halves are arranged so that insertion of the excised circle at an *RS3r* target site will reconstruct a functional w^{hs} gene (Fig. 2). The *P*[*X97*] insertion used here also carried a 2.5 kb *hsp70–GFP* (green fluorescent protein; 20) fusion gene inserted at the *Not*I site to more closely approximate the intended use of this vector; to convey a variety of gene constructs as passengers to a particular target site. Flies that carried *70FLP*3F, a *P*[*X97*, *hsGFP*] insertion on chromosome 2 or 3 and an *RS3r* target site on chromosome 2 or 3, were heat shocked for 1 h during the first 2 days of development. The adults that eclosed were scored for the number of w^+ clones in their eyes as a measure of the efficiency of targeted mobilization following different heat shock conditions. The optimal heat shock temperature was ~37°C in most cases (Fig. 3).

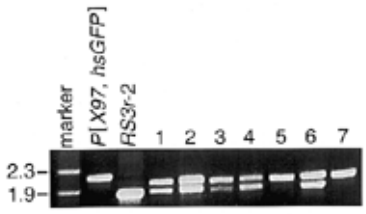

Figure 4. Molecular confirmation of site-specific integration. PCR was used to verify the presence of the expected junction fragments (as indicated in Fig. 2) in the integrants. A sample of the results is shown here. The first three lanes are respectively: molecular weight marker; PCR product using *P*[*X97*, *hsGFP*] genomic DNA as a control template; PCR with w^{1118}; *RS3r*-2 genomic DNA as a control template. Lanes 1–7 are the products of PCR using genomic DNA from seven independent integrants as template. In all cases the 2.0 kb band that indicates site-specific integration is observed. In five of the seven cases the *RS3r* band is also present (see text).

Detecting germline mobilization by reconstruction of w^+

We then used the same *P*[*X97*, *hsGFP*] insertions and *RS3r* target sites to assess targeted mobilization in the germline. As before, flies were heat shocked during the first 2 days of development. The males and females that eclosed were mated *inter se* as three pairs per vial and their progenies then examined for w^+ offspring. All six combinations of donor and target site insertions produced w^+ offspring at rates that varied from 0 to 5% in different experiments, with an overall efficiency of 1.7% for independent events (Table 3). The vials that had w^+ progeny produced an average of 1.9 w^+ progeny each.

We confirmed that the w^+ progeny represented the expected site-specific integration events by two assays. First, we determined whether somatic white mosaics could be generated by further rounds of FLP synthesis. In all cases but one (40/41) mosaics were produced, indicating that the w^+ function could be excised by FLP-mediated recombination. Second, we used PCR to verify that the expected junction fragment was present (Fig. 4). Twenty independent integrants were examined and the expected fragment was amplified in the 19 cases that exhibited mosaicism with FLP. The remaining case, which did not exhibit mosaicism with FLP or produce the expected PCR fragment, also failed to exhibit mosaicism in the presence of the Δ2-3 transposase source.

When a targeted integration event is recovered the fly in which it is found may also carry *70FLP*, an *RS3r* allele without the integrated DNA (as in Fig. 4, lanes 1–4 and 6) and the original or excision remnant form of *P*[X97]. Because *70FLP* and *P*[*X97*] are marked with ry^+ and v^+ respectively, they can be easily removed by crossing. In the course of such crosses any *RS3r* allele will also be removed by segregation from the w^{hs} integrant.

Table 2. Autosome to autosome mobilization of a *white* gene

Donor	Target				
	RS3r-2(75C-D)	*RS3r*-3(82C)	*RS5r*-2B(II)	*RS5r*-3B(38B)	*RS5r*-2A(88B)
P[>w^{hs}>](79B)	1/80	3/80	0/100	0/55	0/91
	1.3%	3.8%	–	–	–

w^{1118} *70FLP*3F; *D P*[>w^{hs}>]/+ males, that also carried a single copy of the indicated target site element either on chromosome 2 or on the chromosome 3 homolog of the donor chromosome, were generated by crossing and heat shocked for 1 h at 34°C during the first 2 days of development. The males that eclosed were mated as one male with two w^{1118} females per vial. Results are reported as (vials with w^+ D^+ progeny)/(total vials). Cytological locations or linkage groups are given in parentheses.

Table 3. Mobilization of a test gene (*hsGFP*) with the vector *P*[*X97*]

Donor	Target site								
	RS3r-4A(51F)			*RS3r*-2(75C-D)			*RS3r*-3(82C)		
	36°C	37°C	38°C	36°C	37°C	38°C	36°C	37°C	38°C
P[*X97, hsGFP*](II)	2/100	1/100	0/100	1/203	3/160	5/139[a]	3/206	3/302	2/200
	2.0%	1.0%	–	0.5%	1.9%	3.6%	1.5%	1.0%	1.0%
P[*X97, hsGFP*](III)	4/95	0/57	5/100	2/98	1/89	5/100	2/95	1/104	0/67
	4.2%	–	5.0%	2.0%	1.1%	5.0%	2.1%	1.0%	–

w^{1118}; *P*[*X97, hsGFP*] males were crossed to w^{1118} *70FLP*3F; *RS3r* females and their progeny heat-shocked for 1 h at the indicated temperature during the first 2 days of developement. When adults eclosed they were mated as two to three pairs/vial. (The chromosome 3 insertion of *P*[*X97, hsGFP*] was balanced over *TM6, Ubx*, so Ubx^+ progeny were selected for this cross). Their progeny were screened for w^+ individuals and the results are reported as (vials with w^+ progeny)/(total vials). Cytological locations or linkage groups are given in parentheses.
[a]One additional vial gave w^+ progeny that did not exhibit mosaicism when crossed back to *70FLP*.

Table 4. Mobilization of *P*[*X97*] using β*2t–FLP* to supply recombinase

Donor	Target site		
	RS3r-4A(51F)	*RS3r*-2(75C-D)	*RS3r*-3(82C)
P[*X97, hsGFP*](II)	1/222	8/181	9/226
	0.5%	4.4%	4.0%
P[*X97, hsGFP*](III)	7/185	2/180	0/122
	3.8%	1.1%	0%

w^{1118} β*2tFLP* males with a single copy of the *P*[*X97, hsGFP*] element and a single copy of the *RS3r* target site (as indicated) were produced by crossing and mated as one male with two to three w^{1118} females. These crosses were screened for w^+ progeny and the results are reported as (vials with w^+ progeny)/(total vials).

The targeted mobilization that we detected in these experiments occurred early in development of the female and male germlines. The number of cells susceptible to mobilization is, therefore, small. We reasoned that if FLP was synthesized at a later stage, when mobilization could occur in a greater number of cells, the targeted integrants might be more efficiently recovered. In females we attempted to increase the efficiency of this method by delaying the heat shock used to induce *70FLP* until later in development, when there are more germline cells. It has been previously observed that such a delay leads to a substantial increase in the rate of mitotic recombination in the female germline (21). Crosses were performed as in Table 3, but the heat shock was performed on day 8 or 9 of development. Because much of the male germline is not susceptible to heat shock at this late stage, most of the targeted mobilization from these crosses should be occurring in females. We observed no increase in the frequency of targeted mobilization in these crosses. Four independent integrants were recovered from 306 vials (1.3%) of the combination of *P*[*X97, hsGFP*] on chromosome 3 and *RS3r*-2(75C-D) with parents that had been heat shocked at 36, 37 or 38°C.

To synthesize FLP at a later stage in the male germline we used a *FLP* gene placed under the control of the promoter of the β*2tubulin* gene (also known as β*Tub*85D; 22). The β*2t* gene is specifically expressed in primary spermatocytes, cells that are in meiotic prophase in the male germline (16). Because these cells undergo their pre-meiotic S phase very quickly after their final mitotic division, every gamete with the target *FRT* represents an independent opportunity for targeted mobilization. The β*2t–FLP* gene is highly efficient in the male germline, producing ~98% excision of the *FRT*-flanked portion of *P*[>w^{hs}>]. In the experiments that used β*2t–FLP* to induce mobilization the desired integrants were easily recovered, arising at an overall rate of 2.4%, slightly higher than in the experiments that used *70FLP* (Table 4). In this experiment there was only one father that produced more than one w^+ offspring. Stocks of 25 putative integrants were established and all 25 exhibited white mosaicism when crossed to *70FLP* and heat shocked early in development, confirming that the w^+ function could be excised by FLP. Each also exhibited white mosaicism in the presence of Δ2-3, confirming that w^+ was located in a *P* element. All 25 were tested by PCR for the fragments diagnostic of targeted integration and all gave the expected products.

Expression pattern of β*2t–FLP*

The data obtained in the β*2t–FLP* experiments present an additional interesting aspect: mobilization to a target site on a heterolog was much more frequent (4.1%) than mobilization to a homolog (0.6%). We believe that this reflects a quirk in expression of the β*2t–FLP* gene construct. Although this gene is probably transcribed in primary spermatocytes, we suspect that translation of its mRNA is delayed until after meiosis. This is a common strategy used in the male germline to delay expression of spermiogenic proteins until after meiosis, when transcription has ceased (23). This can account for the results we observe; integration can occur when donor and target segregate together at meiosis I; when they segregate apart, as homologs do, it cannot. The few cases in which mobilization was detected to a target site on the homolog may occur as a result of a small amount of FLP made pre-meiotically.

We tested this theory by performing additional experiments that used a donor insertion of *P*[*X97, hsGFP*] on a dominantly marked chromosome 3 and a target site on chromosome 2. In 10 of the 11 targeted integrants recovered in this experiment (from 190 vials) the donor and target chromosomes had segregated together in meiosis. A second experiment used the reciprocal combination (*P*[*X97, hsGFP*] on 2 and *RS3r* on 3). Eight integrants were recovered (from 178 vials) and in every case the donor and target chromosomes had segregated together. The most likely explanation is that the majority of β*2t–FLP* mRNA is not translated until after meiosis I.

If we look only at the cases when the donor and target were on heterologous chromosomes, the β*2t–FLP* gene produced a significant increase in efficiency relative to experiments that used

70FLP (4.5 versus 1.7%). Although the cellular efficiency of this method is probably much lower than in the experiments that used *70FLP*, this is more than compensated for by the vast increase in the number of chances for integration to occur.

DISCUSSION

We have shown that site-specific recombination can be used to place genes at specific, pre-selected target sites in the *Drosophila* genome. Targeted gene integration was detected in some experiments by altered linkage of the mobilized gene. In other experiments a donor vector was used (*P*[*X97*]) that generates a w^+ gene when it recombines into a target site, allowing the easy detection of integrants without the necessity of following inheritance of marked chromosomes. We used a variety of protocols to recover targeted integrants. The highest rate of success was obtained when the β*2t–FLP* gene was used to promote mobilization between heterologous chromosomes in the male germline. Targeted integrants were also recovered using a heat shock during the first 2 days of development to induce expression of *hsp70–FLP* fusion genes in the germline. Early developmental heat shocks were used to ensure that all cells of the male germline were in a stage when they were still susceptible to heat shock (8). In the heat shock experiments there was no significant difference in the rates of germline mobilization achieved with 36, 37 or 38°C heat shocks nor was there significant variation in the efficiency of integration when three different locations of the *RS3r* target site were used. There was a barely significant ($P = 0.045$) variation in efficiency between the two *P*[*X97*] donors. Although our experiments did not reveal strong differences in efficiency with different sites, it is certainly conceivable that different donor and target locations might exhibit varying efficiencies of mobilization. For instance, if a donor and target were located close to each other, on the same chromosome or on homologs, the efficiency of integration might be higher because the excised DNA molecule would be more likely to come into contact with the target site.

The efficiency of this method is undoubtedly open to improvement. Altered *FRT*s might be used to prevent re-excision of an integrated DNA (4,13,24). An area that deserves further study is the role of donor–target homology in obtaining integrants. In experiments (not reported here) in which the only homology between the donor episome and chromosomal target was ~200–600 bp surrounding the *FRT*, we recovered targeted integrants only rarely (two from >2000 vials). The successful cases reported here were instances where the donor and target sites bore matching *white* gene sequence. In the experiments diagramed in Figure 1 the donor had ~1.1 kb of homology to the *RS5r* target site and ~4.1 kb of homology to the *RS3r* target and in these experiments (Tables 1 and 2) integration was more efficient at the *RS3r* target site. The experiments in Tables 3 and 4 were also cases where the donor episome and chromosomal target had ~4.1 kb of homology. Homology between donor and target sites might contribute to efficiency by stimulating the maintenance of physical proximity. *Drosophila* pair homologous sequences in mitotic cells (25); this pairing mechanism might bind the episome to the donor when they come into contact, prolonging the opportunity for integrative recombination.

If pairing of homologous sequences does facilitate integration, then manipulations that improve the efficiency of that pairing might improve the efficiency of integration. One way to increase pairing might be to increase the length of time available for pairing of the episomal donor and chromosomal target *FRT*s (8). Primary spermatocytes have a very long meiotic prophase and if FLP were synthesized at this stage, pairing of the episome and target might be more frequent. The particular β*2t–FLP* construct that we used is probably transcribed in primary spermatocytes, but not translated until after meiosis. By altering the 5′- and 3′-untranslated regions of the construct so that it more precisely resembles the β*2tubulin* gene, it may be possible to synthesize FLP in primary spermatocytes, possibly leading to an improved efficiency of targeting.

When targeted integrants are recovered two simple tests typically suffice to confirm that the expected integration event has occurred. First, the putative integrants can be crossed to flies with a heat-inducible *FLP* gene and the progeny heat shocked to produce the somatic mosaicism that indicates excision of the w^+ reporter gene (7). The vast majority of putative integrants recovered in the work reported here were re-excised by FLP (79/83 tested). As a second test, PCR can provide rapid molecular confirmation of the integration event. Forty four of the integrants that produced white mosaicism with FLP were tested by PCR and the expected junction fragment was detected in all 44 cases. The exceptional flies, in which FLP-mediated integration was not confirmed by genetic or molecular tests, are potentially quite interesting. Some may represent cases of FLP-mediated integration at genomic sites that resemble *FRT*s. Sequences that are used as low efficiency target sites by the Cre recombinase have been found in the yeast genome (26). It is also possible that occasional rare integrations occur by mechanisms unrelated to FLP. In other cases such flies might arise from mutational events occurring at an *RS3r* target site. The 3′-portion of *white* carried by *RS3r* is sufficient to encode a functional white protein if expressed and similar effects have been observed previously, also at low frequency (17).

The ability to place a variety of gene constructs at a specific site and in a specific orientation allows the design of experiments that were previously impractical or impossible. For instance, it should be possible to investigate subtle effects on gene expression when a series of variants are placed at the same site, because such effects will not be swamped by the effects of chromosomal position. This technique also provides the potential for the examination of particularly interesting position effects, such as variegated effects (27) or those that confer an unusual pattern of expression (28). A variety of potential insulators, enhancers and promoters could be tested for their resistance or susceptibility to position effects by placing them, one by one, at a specific chromosomal *FRT*. One method for placing two genes at a site has been previously described (29). FLP-mediated mobilization allows for the placement of an unlimited number of constructs at a given site.

Finally, the demonstration that FLP can mediate DNA excision and re-integration suggests the possibility that it may be used as a method of directly integrating, at a chromosomal *FRT* target site, DNAs injected into embryos. This would bypass the steps involved in constructing and transforming P elements with the donor DNA. This might also allow the transformation of DNA segments that are too large to transform in transposon vectors. Reports that injected DNAs can be used as templates for *P* transposase-induced gap repair show that the injected DNAs can pair with homologous chromosomal sequences, supporting the possibility that this technique is achievable (30,31).

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