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FLP-mediated recombination in the vector mosquito, *Aedes aegypti*

Alison C.Morris, Theresa L.Schaub and Anthony A.James
Department of Molecular Biology & Biochemistry, University of California, Irvine, CA 92717, USA

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ABSTRACT

The activity of a yeast recombinase, FLP, on specific target DNA sequences, FRT, has been demonstrated in embryos of the vector mosquito, *Aedes aegypti*. In a series of experiments, plasmids containing the FLP recombinase under control of a heterologous heat-shock gene promoter were co-injected with target plasmids containing FRT sites into preblastoderm stage mosquito embryos. FLP-mediated recombination was detected between (i) tandem repeats of FRT sites leading to the excision of specific DNA sequences and (ii) FRT sites located on separate plasmids resulting in the formation of heterodimeric or higher order multimeric plasmids. In addition to FRT sites originally isolated from the yeast 2 μm plasmid, a number of synthetic FRT sites were also used. The synthetic sites were fully functional as target sites for recombination and gave results similar to those derived from the yeast 2 μm plasmid. This successful demonstration of yeast FLP recombinase activity in the mosquito embryo suggests a possible future application of this system in establishing transformed lines of mosquitoes for use in vector control strategies and basic studies.

INTRODUCTION

Mosquitoes are responsible for transmitting the highest number of cases of parasitic diseases (1). Furthermore, they transmit the greatest variety of pathogens including viruses, protozoans, and metazoans, specifically filarial worms. This pre-eminence in numbers of disease cases caused and variety of disease transmitted has made mosquitoes the recent focus of studies examining the biochemical and molecular bases of vector competence. These efforts have shed new light on gene expression in mosquito organs such as the salivary glands (2,3) where significant interactions with pathogens take place. In addition, new research has demonstrated unexpected interactions between vectors and vertebrate hosts (4,5), and raised the possibility of novel control mechanisms for transmission of parasitic diseases (6,7). These studies have established a clear need for techniques that would allow the introduction of genes and gene constructs into mosquitoes. These techniques would further the molecular analysis of endogenous mosquito genes involved in vector competence and other vector properties such as bloodfeeding. In addition, transformation techniques would permit the introduction of exogenous genes whose activities could be targeted to interfere with parasite growth and development in the vector resulting in reduced vector competence. Ultimately, transformation techniques would result in the production of transgenic mosquitoes that can be used in release programs to decrease the transmission of parasitic diseases.

There have been several reports of the successful transformation of mosquitoes (6,8,9). Each of these efforts was based on the premise that the P transposable element of *Drosophila melanogaster* would mobilize and integrate into the mosquito genome. The results in all of these reports were similar, transformed lines containing exogenous constructs were obtained after microinjecting embryos with plasmid DNA. However, transformed lines were obtained at frequencies of less than 0.1% of the embryos injected and transformation appeared not to be P-element mediated, but to result from illegitimate recombination. The low frequency of integration precludes the use of this approach for the routine transformation of mosquitoes. However, illegitimate recombination events similar to those observed in these efforts could be utilized effectively if the integrated sequences carried targets for a heterologous, high frequency, site-specific recombination system. Such a target DNA sequence could serve as a docking site for the high frequency integration of exogenous DNA.

Research is currently underway in our laboratory to investigate the activity in mosquito embryos of a site-specific recombination system encoded by the 2 μm plasmid of the yeast, *Saccharomyces cerevisiae* (10). This plasmid is found in many yeast strains, carries two inverted recombination target sites (FRT), and codes for a recombinase protein (FLP) that recognizes specifically the target sites. In the 2 μm plasmid, FLP recombinase will catalyze recombination between the FRT sites inverting the DNA sequences between them. FLP is the only protein required for this activity. The autonomy of the FLP protein, its eukaryotic origin, and the fact that it can catalyze intramolecular and intermolecular recombination in *D. melanogaster* (11,12), *Escherichia coli* (13) and mammalian cells (14), has led us to investigate the possibility of using it as a gene transfer system in mosquitoes. We report here that FLP-mediated, site-specific recombination can occur in the vector mosquito, *Aedes aegypti*. In an excision assay, we show that intramolecular recombination can be very efficient. In another assay, FLP-mediated recombination can result in the formation of heterodimeric plasmid molecules, an intermolecular recombination event functionally equivalent to integration. Both endogenous and synthetic FRT sites will serve as substrates for recombination.

MATERIALS AND METHODS

Mosquitoes

All mosquitoes used in these experiments were the Rockefeller strain of *Ae. aegypti*. Mosquitoes were reared using standard procedures established in our laboratory.

Plasmids used

Two classes of plasmids, source and target plasmids, are used in the experiments. Source plasmids serve as the source of FLP recombinase in the assays. The source plasmid, pP[ry$^+$hsFLP], was the same for all of the experiments and was a gift of K. Golic (University of Utah). This plasmid codes for the FLP recombinase under control of the *D. melanogaster* heat shock 70 gene promoter. Ideally, this plasmid would allow us to vary the amount of FLP recombinase in a given series of experiments depending on whether or not animals were heat shocked.

The target plasmids used to evaluate intramolecular recombination were constructed for an excision assay such that a unique restriction endonuclease site is flanked by tandem FRT sites. Intramolecular recombination between the tandem FRT sites excises the intervening DNA, removes the unique restriction endonuclease site, and yields a product plasmid that is resistant to digestion with the corresponding restriction endonuclease. Methods for detecting these plasmids are described below.

The target plasmid used in the excision assays described in this report is a heterodimeric plasmid composed of a single pBR322 molecule (15) and a single pACYC184 molecule (16) (Fig. 1). These plasmids are compatible and share homology only in the region of their respective tetracycline-resistance genes. A 670bp *Bam HI/Hind III* fragment from the yeast 2 μm plasmid containing FRT sites was excised from the plasmid pDW (11) and inserted into the tetracycline-resistance genes of pBR322 and pACYC184. These insertions are accompanied by a 346bp deletion in the tetracycline-resistance gene between its unique *Bam HI* and *Hind III* restriction endonuclease sites. The heterodimeric plasmid was formed in a FLP-mediated, intermolecular recombination event in experiments described in the RESULTS. The heterodimeric plasmid was isolated, its structure verified by restriction endonuclease digestion, and large amounts purified by CsCl density gradient centrifugation. This particular target plasmid has a unique *Pvu I* site in the pBR322 moiety flanked by the FRT sites.

The target plasmids used to evaluate intermolecular recombination were also constructed from pBR322 and pACYC184 (Fig. 2). Monomeric target plasmids with yeast 2 μm plasmid sequences containing FRT sites were those described above. Additional plasmids were constructed that contain paired oligonucleotides encoding synthetic FRT sites. Minimal 34bp FRT sites (Fig. 3) flanked by *Bam HI* and *Sal I* restriction endonuclease sites were synthesized. The synthetic sites were made by annealing complementary 54bp oligonucleotides. The synthetic FRT sites were inserted into the tetracycline-resistance genes of pBR322 and pACYC184 using the corresponding unique restriction sites. In this case, the cloning was accompanied by a 276bp deletion of the tetracycline-resistance gene.

In addition to wild-type sites, two mutant FRT sites were synthesized and cloned. It was expected that these sites might stabilize integrant formation (17). One mutant has a CA transversion at the −10 position. The second FRT site has a GT transversion at the +10 position. Recombination between these two point mutations will result in a wild-type FRT site and an FRT site with mutations at both positions, −10 and +10. This double mutant requires elevated levels of FLP protein to facilitate recombination, hence stabilizing the recombinant. The synthetic sites also are flanked by *Bam HI* and *Sal I* sites and were cloned into pACYC184 and pBR322. During cloning, the synthetic sites are cleaved to yield 46 bp targets with the following structures:

5′-**GG-ATC-CGA-AGT-TC***A*-TAT-ACT-TTC-TAG-AGA-ATA-GGA-ACT-TCG-**TCG-AC**-3′

5′-**GG-ATC-CGA-AGT-TCC-**TAT-ACT-TTC-TAG-AGA-ATA-T**G**A-ACT-TCG-**TCG-AC**-3′

Point mutations are underlined. The *Bam HI* and *Sal I* sites are in bold. The structure of all constructs was verified by dideoxy sequencing.

Micro-injection and plasmid recovery

Micro-injection was performed on 1−2½ hour old *Aedes aegypti* embryos as described by Morris *et al.*, (9). The DNA solution (500pl) introduced into the embryos consisted of source and target plasmids at a total concentration of 1 mg/ml and at a ratio of 1:1 (excision assay) or of 1:1:1 (integration assay), in 5 mM KCL, 0.1 mM sodium phosphate, pH 6.8. In control experiments in which only target plasmids were injected, a total plasmid concentration of 1 mg/ml was used.

After injection, embryos were placed on moist filter paper and maintained under standard rearing conditions for approximately 18 hours. The filter paper carrying the embryos was then placed inside a 50 ml Falcon centrifuge tube and incubated in a 40°C water bath for 1 hour. The embryos were then allowed to recover under rearing conditions for 2−3 hours.

Low molecular weight DNA was recovered from injected embryos as described by Hirt (18). 50−100 embryos were extracted in 100 μl of Hirt lysis buffer. In the excision assay, the extracted DNA was resuspended in 20 μl TE (10mM Tris, 1mM EDTA, pH=7.5). 10 μl of this sample was digested with *Pvu I* at 37°C. The remaining 10 μl of DNA was used as an uncut control and otherwise treated identically as the digested sample. Cut and uncut samples were ethanol precipitated and resuspended in 5 μl TE. Each sample was transformed by means of electroporation into 40 μl of electroporation competent *E. coli* DH5α cells prepared as described by Dower *et al.*, (19). Electroporation was carried out in a Biorad 0.2 cm electroporation cuvette with a pulse of 2.2 kV, a 25 μF capacitor and 200 ohm parallel resistor to give transformation efficiencies of 10^8-10^9 transformants/μg DNA. Transformants were selected on LB agar plates with chloramphenicol (30 μg/ml).

In the integration assay, the total low molecular weight DNA extracted from 50−100 embryos was resuspended in 5μl of TE and transformed into *E.coli* DH5α cells by electroporation. Transformants were selected on LB agar plates with ampicillin/chloramphenicol (100μg/ml and 30μg/ml respectively).

Plasmid DNAs were prepared from individual chloramphenicol/ampicillin resistant colonies using the method of Holmes and Quiqley (20). DNA is resuspended in 40 μl TE and 5−10 μl digested with the restriction endonuclease enzymes *Pvu I* and *Nde I*, both of which cut in the pBR322 moiety to release a 1.44 kb fragment. Digestion of a heterodimer produces two DNA fragments of approximately 7.82 kb and 1.44 kb. Higher order multimers will contain these fragments and an additional fragment of approximately 3.25 kb. Co-transformed monomeric plasmids produce only the fragments corresponding to 3.25 kb and 1.44 kb and the undigested pACYC184 monomer. These fragments are easily visualized by subjecting them to 0.8% agarose gel electrophoresis (Fig. 4).

RESULTS

The excision assay is based on the expectation that FLP-mediated recombination will excise from a target molecule those sequences flanked by tandem FRT sites (Fig. 1). As described in the construction of the target plasmids, we arrange it so that the excision event removes a unique restriction site, *Pvu I*, from a plasmid to yield product plasmids resistant to restriction enzyme digestion. Product plasmids are detected following digestion with *Pvu I* and transformation of *E. coli* by a sample of the recovered DNA. The amount of recombination observed is expressed as the percentage of chloramphenicol-resistant colonies resulting from the *Pvu I*-cut DNA sample divided by the number resulting from the undigested sample. A number equal to 100% indicates recombination of all target plasmids. Control experiments for the excision assay include microinjection without source plasmids and transformation of *E. coli* with source and target plasmids without microinjection and recovery from mosquito embryos.

The excision assay will indicate whether or not FLP-mediated, site-specific recombination can occur in mosquito embryos and if so, can the frequency be controlled by application of heat shock. As seen in Table 1, FLP-recombinase will catalyze excision of a fragment of DNA containing a unique restriction site resulting in restriction enzyme-resistant plasmids. An average of 60% of all plasmids recovered have been recombined in the heat-shock stimulated experiments and approximately 32% in the non-heat shock experiments. In experiments with a different target plasmid, we have seen 100% recombination of targets (A.C. Morris and A.A. James, unpublished). A sample of the plasmids that escaped enzyme digestion were examined by miniprep analysis to verify their structure and 100% (72/72) had recombined to eliminate the *Pvu I* site. Colonies recovered in the experiments with no source plasmid were shown not to have recombined out the *Pvu I* restriction site (52/52). The level of recombinants in the non-heat shock group is high, but we have determined recently that there is significant constitutive expression afforded by the *D. melanogaster* heat shock 70 gene promoter in mosquito embryos in the absence of heat shock (A.C. Morris and A.A. James, unpublished). In a control experiment where source and target plasmids were transformed into *E. coli*, a sample of colonies (n=18) recovered from the enzyme-digested group were all shown to be non-recombinants that escaped digestion. These data indicate that FLP-mediated recombination of target plasmids can occur in mosquito embryos.

Like the excision assay, the integration assay is based on recovering target plasmids from mosquitoes following microinjection (Fig. 2). The target plasmids are monomers of pBR322 and pACYC184 containing the FRT sites derived from the yeast 2 μm plasmid, the wild-type synthetic FRT sites, or the mutant synthetic FRT sites. Recombination produces a heterodimeric molecule capable of conferring resistance in *E. coli* to both ampicillin (pBR322) and chloramphenicol (pACYC184) following recovery of DNA from mosquito embryos and transformation. The FLP source plasmid is the same as used in the excision assay. These experiments are complicated by the fact that co-transformation of *E. coli* with the respective monomeric units produces the double antibiotic-resistant phenotype expected of the heterodimeric recombination product.

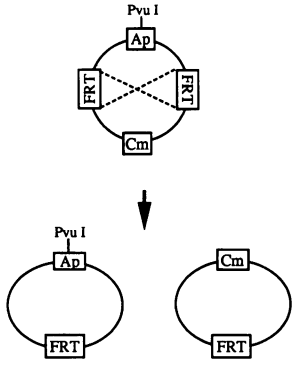

Fig. 1. Excision assay target plasmid (top) and its predicted products following FLP-mediated recombination (bottom). The relevant features of the target plasmid are that it contains a unique restriction site, *Pvu I*, flanked by tandem repeats of FRT sites. Abbreviations: Ap, ampicillin resistance gene; Cm, chloramphenicol resistance gene; FRT, recombination target sequence; *Pvu I*, unique restriction endonuclease cleavage site.

Table 1. FLP-mediated excision in the mosquito embryo

FLP source[a]	+ heat shock[b]	− heat shock[b]	N[c]
equimolar	60.2	32.0	3
none	2.9	0.37	2

a. Indicates whether FLP source plasmids were included in the microinjection and their relative concentrations.
b. Indicates the average percentage of plasmids recovered that escape *Pvu I* digestion in the assay described in the Materials and Methods.
c. Indicates number of repeat experiments.

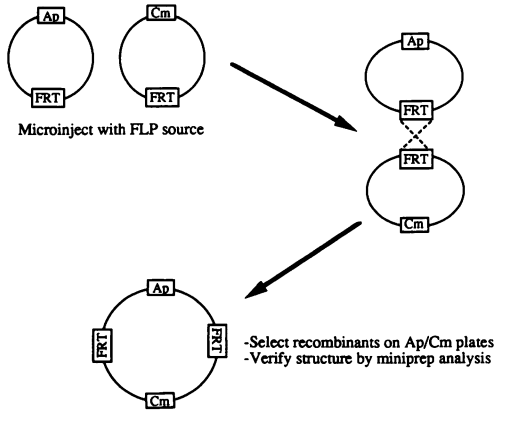

Fig. 2. Integration assay target plasmids (top) and the predicted heterodimeric product (bottom) following FLP-mediated recombination. The two target plasmids undergo FLP-mediated recombination to give rise to a heterodimeric plasmid capable of conferring resistance to chloramphenicol and ampicillin following transformation of *E. coli*. Abbreviations as in Fig. 1.

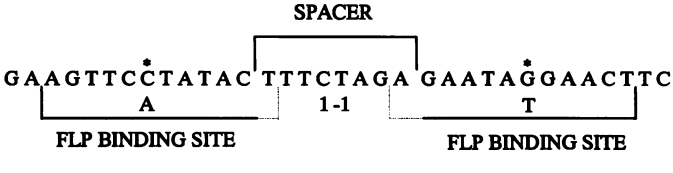

Fig. 3. Minimal FLP binding site required for FLP-mediated recombination in mosquitoes. It has been demonstrated that an FRT site with two specific point mutations (position 10 in each of the FLP binding sites flanking the spacer region indicated by an asterisk above and the nucleotide substitution below) requires a 50-fold increase in FLP activity to catalyze recombination with a similar or wild-type sequence (after Senecoff *et al.*, [17]).

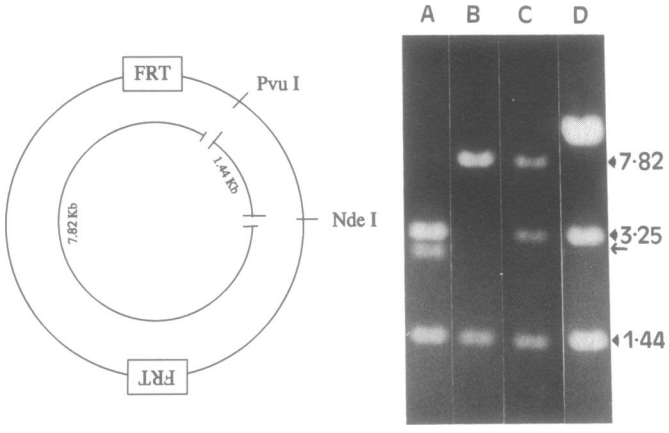

Fig. 4. DNA miniprep analysis of double-resistant colonies arising from the integration assay. Diagrammatic representation of heterodimer arising from FLP-mediated recombination of target plasmids (left). *Nde I/Pvu I* digests of integration and excision assay products (right). (A) Co-transformed Cm^R and Ap^R target plasmids. (B) Ap^R/Cm^R heterodimer. (C) Ap^R/Cm^R trimer composed of 2 Ap^R and 1 Cm^R units. (D) Ap^R/Cm^R multimer (>3). Numbers refer to fragment size in kilobases. Small arrow indicates uncut pACYC184. The large fragment in lane D can not be accurately sized on this gel.

Table 2. Integration recombination events in the mosquito, *Aedes aegypti*

FLP source[a]	Heat shock[b]	Yeast FRT[c]	Wild-type synthetic site[c]	Mutant synthetic site[c]	No FRT site
equi-	+	7 (80)	5 (82)	15 (80)**	0 (72)
molar*	−	4 (80)	6 (80)	8 (80)	0 (71)
10^{-2}	+	2 (79)	1 (78)	1 (84)	−
	−	0 (73)	0 (84)	0 (81)	−
none	+	1 (78)	0 (76)	1 (162)	−
	−	2 (76)	0 (74)	4 (131)	−

Numbers indicated are recombinant dimers or higher-order oligomers verified by DNA miniprep analysis of a sample of recovered double-resistant transformants. Figures in parentheses indicate the total number of minipreps analyzed in each experiment.

a. Ratio of FLP source plasmid co-injected with target plasmids.

b. Designates whether embryos were heat-shocked during protocol.

c. Indicates origin of FRT sites in plasmids.

* Indicates all values in this group are significantly different ($P < .05$ by Fisher 1-tail or Mantel-Haenszel analysis) than values in the 10^{-2} and no FLP source groups.

** indicates that the combined value of the heat shock and non-heat shock experiments were significantly different ($P < .05$, Mantel-Haenszel analysis) from the combined values of the wild-type synthetic site experiments).

− Not determined

Therefore, all data reported here are dimers or higher order multimers that have been verified by miniprep DNA analysis (Fig. 4). Control experiments for the integration assay consist of the microinjection procedures without the FLP source plasmid, and direct transformation of *E. coli* with source and target plasmids without microinjection and recovery from embryos.

The number of dimer and higher-order multimers observed in the integration assay and verified by miniprep analysis was low. Therefore the significance of the data was statistically evaluated. In cases where we were comparing one or more observed dimers in one experiment with an experiment where no (0) dimers were observed, we used the Fisher exact (1-tail) test. All other evaluations were done with the Mantel-Haenszel test. These tests are part of a statistical package (EpiInfo) distributed through the CDC (Atlanta). In all cases only values of $P < 0.05$ were considered significant.

The analysis of intermolecular recombination with our integration assay provides evidence that these events do occur. As seen in Table 2, heterodimeric plasmids resulting from FLP-mediated recombination were detected in experiments where the FLP source plasmid was present in equimolar amounts with the target plasmids. We did not detect any significant difference between the experiments with the FLP source plasmid with or without heat shock. Apparently the previously-detected constitutive level of FLP expression is sufficient to catalyze recombination in non-heat shock conditions. We did several experiments with the FLP source plasmid at a 100-fold lower concentration and were unable to detect integration events in numbers that were significantly higher than our control experiments where no source plasmids are supplied. Interestingly, we did recover a few apparently recombinant plasmids in our control experiments where target plasmids were microinjected without source plasmids. Since we saw no recombinants (0/54 minipreps analyzed) in experiments where target and source plasmids were transformed into *E. coli* without prior microinjection, it is possible that the target plasmids can interact with one another in some event mediated by the mosquito embryo. We also detected a small number ($n = 7$ of an overall total of 1754 minipreps examined in all experiments) of plasmids whose structure after enzyme digestion was not easily interpreted but which did not represent heterodimers or constituent monomers. We did not include these in our analysis. Control experiments were done with source plasmids and pBR322 and pACYC184 monomers with no FRT sites. No dimers were detected in a sample ($n = 143$) of double resistant colonies subject to miniprep analysis.

The experiments with the synthetic FRT target sites produced results similar to those obtained using the sites derived from the yeast 2 μm plasmid. This is taken to indicate that synthetic FRT sites can be used in experiments to evaluate FLP-mediated recombination in heterologous systems. The effects of the mutant FRT site were significantly different from those of the wild-type site ($p = 0.027$, Mantel-Haenszel, Chi square = 4.89 comparing the combined data from both heat-shock and non heat-shock experiments) yielding a higher number of dimers. However the effect was not as large as anticipated. Further experiments will be required to determine if the mutant sites will stabilize integration in a practical sense.

DISCUSSION

The data presented here provide compelling evidence that FLP-mediated recombination can occur in mosquito embryos. Furthermore, this recombination can be intermolecular, an event that is functionally identical to an integration event. FLP-mediated recombination can be very efficient as evidenced by the excision assay and can occur with synthetic FRT target sites.

The FLP-FRT site-specific recombination system has been demonstrated to function in a number of heterologous organisms (11−14). We have shown here that it can function in the embryos of the vector mosquito, *Ae. aegypti*. A number of conclusions can be drawn from this work. FLP-mediated events evaluated by the excision assay can be very efficient. The majority of target plasmids can be recombined in FLP-mediated events. This assay

could detect differences between induced and constitutive levels of the recombinase and, surprisingly, constitutive levels of FLP expression gave a high frequency of excision. These data indicate that the excision assay can be a sensitive measure of FLP activity in the mosquito embryo.

The frequency of integration events is difficult to quantify because of a number of complicating factors. Perhaps the most significant factor is that heterodimers formed during the integration assay are now substrates for FLP-mediated excision events. Our excision experiments have shown that these events occur at high frequencies. As substrates become more dilute as a function of diffusion in the embryo, the most likely FLP-mediated events to occur will be those that involve two target sites in the same molecule. Thus, we may miss a large number of integration events that produce dimers in the mosquito because they recombine to constituent monomers before we have a chance to assay them. An additional factor to consider is that variations in transformation efficiencies could affect the ratio of double-resistant colonies arising from co-transformation to those arising from recombinant heterodimers.

We attempted to address the problem of integrant stability with several different approaches. In our first efforts, we tried to vary the amount of FLP-recombinase present in the embryo. This was achieved initially by using an inducible promoter. However, as was demonstrated, non-heat shock expression is sufficient to catalyze substantial recombination and could destabilize at least 30% of integrants. In a different effort, we reduced the level of the FLP source plasmid by 100-fold to see if this would result in a higher recovery of integrants. We did not see this, and we do not know if that is because there are fewer initial integrants formed or if the activity is still high enough to resolve all integrants. It is most likely that some combination of these factors occurs.

Our second approach to stabilizing integrant formation was to mutate the FRT site. Studies on the recombination potential of FRT sites with mutations in the 13bp repeats flanking the spacer region have revealed decreases in recombination activity dependent on specific mutations (17). Increased levels of FLP recombinase were required to resolve plasmids containing mutant FRT sites. We constructed similar single point mutations in our synthetic FRT sites. Recombination between the single mutant sites would produce a heterodimeric molecule with one wild-type site and one double-mutant site. The double-mutant site has the lowest ability to serve as a target site when compared with either the wild-type or single-mutant sites. In principle, this should lead to an increased stability of integrants formed by recombination between two single mutant sites as compared to other possible combinations. Although we did recover a statistically significant higher number of dimer molecules using mutant target sites, the number was not as large as expected (we expected a 10–50 fold difference based on the yeast experiments of Senecoff *et al.*, [17]). This suggests that either the level of FLP activity is great enough such that the requirements for increased FLP activity to resolve integrants to constituent monomers is fulfilled, or that point mutations do not influence the level of FLP activity required for recombination in the mosquito.

The results of our integration analysis are consistent with the observations of Huang *et al.*, (13), in the FLP-mediated, FRT site-specific targeting of exogenous DNA into the *E. coli* chromosome. They demonstrated that mutant FRT sites did not increase stability of integrants. Furthermore, using a strategy of integrating FRT sites into the chromosome by site-specific recombination with an FRT site previously introduced into the genome by Tn5 transposition, they demonstrated that a low level of FLP-expression from a leaky promoter was sufficient to destabilize integrated donor DNA. The only effective way to stabilize integrants was to limit expression of FLP recombinase to a few minutes after addition of the donor DNA. This was achieved by controlling FLP expression with the heat-inactivated repressor, C1857 from phage λ. In the absence of a repression system for the expression of the FLP recombinase in mosquito embryos, alternate strategies such as supplying FLP as either a translatable mRNA or as a purified protein are required.

Several significant questions remain to be answered before the FLP-FRT system can be used to routinely introduce exogenous DNA into the mosquito genome. These include determining conditions that will increase the frequency and stability of FLP-mediated integration events, and determining if recombination will proceed efficiently with an FRT site integrated into the genome of the mosquito. The stability of an FRT docking site introduced into the genome by illegitimate recombination remains to be determined. However, short term stability of integrated docking sites and FLP-mediated events may be sufficient to assess the effect of specific gene constructs on vector competence and development. *In vivo* studies of this nature would require that FLP mediated integration events occur at the level of the germline. Since these experiments were carried out using whole embryos, germline specific events cannot be distinguished in this instance. However, the results presented here are sufficient to stimulate further work in this area. Ultimately, this system may prove to be an invaluable tool in further strategies for producing transgenic mosquitoes.

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