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Purified *mariner* (*Mos1*) transposase catalyzes the integration of marked elements into the germ-line of the yellow fever mosquito, *Aedes aegypti*

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Abstract

Derivatives of the *mariner* transposable element, *Mos1*, from *Drosophila mauritiana*, can integrate into the germ-line of the yellow fever mosquito, *Aedes aegypti*. Previously, the transposase required to mobilize *Mos1* was provided in trans by a helper plasmid expressing the enzyme under the control of the *D. psuedoobscura* heat-shock protein 82 promoter. Here we tested whether purified recombinant *Mos1* transposase could increase the recovery of *Ae. aegypti* transformants. *Mos1* transposase was injected into white-eyed, kh^w/kh^w, *Ae. aegypti* embryos with a *Mos1* donor plasmid containing a copy of the wild-type allele of the *D. melanogaster cinnabar* gene. Transformed mosquitoes were recognized by partial restoration of eye color in the G_1 animals and confirmed by Southern analyses of genomic DNA. At *Mos1* transposase concentrations approaching 100 nM, the rate of germ-line transformants arising from independent insertions in G_0 animals was elevated 2-fold compared to that seen in experiments with helper plasmids. Furthermore, the recovery of total G_1 transformants was increased 7.5-fold over the frequency seen with co-injected helper plasmid. Southern blot analyses and gene amplification experiments confirmed the integration of the transposons into the mosquito genome, although not all integrations were of the expected cut-and-paste type transposition. The increased frequency of germ-line integrations obtained with purified transposase will facilitate the generation of *Mos1* transgenic mosquitoes and the application of transgenic approaches to the biology of this important vector of multiple pathogens.

1. Introduction

The development of genetic transformation systems for the yellow fever mosquito, *Aedes aegypti* (Coates et al., 1998; Jasinskiene et al., 1998), has resulted in significant advancements in the ability to assess gene function and expression in this important vector species (Coates et al., 1999). Furthermore, transformation provides a basic tool needed to generate transgenic mosquitoes that may be used to control the transmission of disease-causing pathogens (James et al., 1999; Olson et al., 1996). The frequency of germ-line transformation is not yet at the level enjoyed by *Drosophila* workers, and this combined with other difficulties associated with rearing mosquitoes provides a substantial incentive towards improving present transformation protocols.

Previous successful efforts (Coates et al., 1998; Jasinskiene et al., 1998) to transform *Ae. aegypti* with the *Mos1 mariner* (Medhora et al., 1991) and *Hermes* transposable elements (O'Brochta et al., 1996), utilized a helper plasmid that produces a transposase protein under the control of a heat-shock gene promoter. In these experiments, the level of transcription, mRNA stability and extent of translation into active protein was not known. In the absence of this knowledge, we reasoned that purified transposase protein might catalyze integration at a higher frequency than that seen with the use

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of a helper plasmid. The use of purified protein removes the need for transcription and translation from a plasmid template and alleviates potential problems such as promoter utilization, mRNA stability and codon bias. Purified recombinant *Mos1* transposase (Tosi and Beverley, 2000) was found to integrate marked *Mos1* elements at twice the transformation rate observed when using a helper plasmid, and the recovery of total transformed animals was 7.5-fold higher.

2. Materials and methods

Preblastoderm embryos of the white-eyed strain, kh^w/kh^w (Bhalla, 1968), were injected as previously described (Jasinskiene et al., 1998), except that purified *Mos1* transposase was used instead of a helper plasmid. The donor plasmid, pM[*cn*] (Coates et al., 1998), contains a modified *Mos1* element carrying a wild-type copy of the *D. melanogaster cinnabar* (*cn*) gene that partially complements the kh^w mutation restoring some color to the adult eyes (Cornel et al., 1997; Coates et al., 1998). Transposase protein was prepared as described in Tosi and Beverley (2000). The transposase was supplied as a 50 ng/μl stock in buffer (10% glycerol, 25 mM Tris–HCl pH 7.6, 50 mM NaCl, 0.5 mM DTT, 5 mM $MgCl_2$) and some aliquots were diluted to 0.5 ng/μl in the same buffer. The transposase solutions were mixed in a 3:1 ratio with a 2 μg/μl solution of pM[*cn*] in injection buffer (Jasinskiene et al., 1998). Final effective concentrations of transposase were determined to be 0.38 and 38 ng/μl for the 0.5 and 50 ng/μl stock solutions, respectively. The final plasmid concentration of 0.5 μg/μl was identical to that used in the previous transformation experiments (Coates et al., 1998; Jasinskiene et al., 1998). The injection mixes were aliquoted into 5 μl amounts, frozen in a dry ice/ethanol bath, and stored at −70°C until used. During injection procedures, mixtures were kept on ice for no more than 3 h, and at room temperature in injection needles for no longer than 2 h.

The husbandry and genetic crosses of the surviving injected mosquitoes were performed as described in Jasinskiene et al. (1998). G_0 adult males were mated individually with multiple kh^w/kh^w females and used to establish single-founder families. Surviving G_0 adult females were mated in pools with multiple kh^w/kh^w males. G_1 progeny resulting from potentially transformed adults were screened for the appearance of eye color. Positive G_1 individuals were used to establish transgenic families. Genomic DNA was isolated from these individuals, prepared for Southern analyses, and probed with the radio-labeled 4.7 kb fragment of *D. melanogaster* DNA carrying the cn^+ gene as previously described (Coates et al., 1998; Jasinskiene et al., 1998). "Inverse polymerase chain reactions" (IPCR) were performed to isolate the junction fragments of lines 52 and 52.2 and these were analyzed as described in Coates et al. (1998). Transformation rates are a measurement of how many independent transposition events occurred. These rates are calculated as a percentage of the number of independent transposon integrations divided by the total number of fertile G_0 adults. The frequency of transformed animals measures the total number of transformed animals recovered and also is expressed as a percentage. This frequency is determined by dividing the total number of transformed animals observed by the total number of G_1 progeny screened.

3. Results and discussion

We compared the survival rates, transformation rates, and frequency of recovery of G_1 transformants of *Ae. aegypti* injected with a *Mos1* transposon donor and either purified transposase or transposase provided by a helper plasmid. The survival rates of injected embryos to the adult stage were comparable among the animals injected with helper plasmid followed by heat shock or those injected with transposase at two different concentrations (14% vs 16%; see Table 1; Coates et al., 1998). These results indicate that any differences in the microinjection procedures between the two experiments, for example, the heat-shock regimen required when using a helper plasmid, or the differences in buffer composition, appear to not affect the overall survival rate. The levels of adult fertility were also comparable to those observed in the previous experiments (data not shown).

A total of 81 individual families were established with fertile G_0 founder males following injection with 0.38 ng/μl transposase and the transposon donor plasmid (Table 1). One male-founder family, 23, produced colored-eyed G_1 progeny, yielding a transformation rate of 1.2%. The 111 surviving G_0 females were combined and mated in several pools from which a single transformed family, P12, was isolated. Because we do not know how many of the G_0 females in each pool were fertile, these data are tabulated independently from those obtained with the single male-founder families. In addition, the observed transformation rate, 0.9%, is most likely an underestimate. The detection of one transposon integration in each of the groups, the male single-founder families and the female pools, does not allow for rigorous comparisons of transformation rates between this experiment and those using helper plasmids. However, both rates are lower than those obtained with the helper plasmid experiments.

Thirty-five male-founder families were recovered following injection with 38 ng/μl transposase and donor plasmid (Table 1). Two of these families, 52 and 53, produced colored-eyed progeny in the G_1 generation. Upon further analysis, it was observed that family 52 produced G_1 individuals with two different eye color phenotypes, and that these segregated in subsequent gen-

Table 1
Summary of data from the use of *Mos1* transposase from different sources to generate *Ae. aegypti* transformants

	Helper plasmid[a]	0.38 ng/ul transposase	38 ng/ul transposase
Number of embryos injected	1625	1405	702
Number of adult survivors	231 (14%)	224 (16%)	115 (16%)
Transformation rates[b]	3/69 ♂ (4.3%)	1/81 ♂ (1.2%)	3[c]/35 ♂ (8.6%)
	2/110 ♀ (1.8%)	1/111 ♀ (0.9%)	2/50 ♀ (4%)
G_1 transformants	38/20,728 (0.2%)	5/23,407 (0.02%)	121/8139 (1.5%)

[a] These data are taken from Coates et al. (1998).
[b] Transformation rates are calculated as the number of single founder families that produce transformed progeny (♂, male families) or number of independent lines derived from total number of pooled females (♀, female families). The numbers for the transformation rates in males include only fertile males whereas the rates for females are calculated from the total number of females and thus may underestimate true rates.
[c] This rate is calculated taking into account the two separate insertions in family 52.

erations. These results indicate that two independent *Mos1* integration events occurred in different germ-line progenitor nuclei of the family 52 founder male. These two events could have occurred contemporaneously, or one may have resulted from remobilization of a primary insertion. The transposase still may have been present following the initial event and caused a remobilization. A number of true-breeding stocks were established for both eye-color phenotypes, and one of each, 52 and 52.2, were analyzed further. The transformation rate, 8.6%, is based on these three independent events (52, 52.2 and 53), and is twice that seen with the use of a helper plasmid (4.3%; Coates et al., 1998). Several pools were established with the 50 surviving G_0 females, and these yielded an additional two families, P1 and P3. Again, uncertainty in female fertility provides a minimum transformation rate of 4%. These data indicate that purified transposase at 38 ng/μl produces higher transformation rates than the lower concentration and than with the use of the helper plasmid.

The transgenic families generated with 38 ng/μl of transposase had a 7.5-fold higher frequency of transformed progeny than what was observed using the *Mos1* helper plasmid (Table 1; Coates et al., 1998). Most likely this increased frequency results from integration of the transposon into chromosomes of germ-line nuclei early in their development and gives rise to a large clone of transformed progeny cells that go on to differentiate into gametes. Integration mediated by a helper plasmid requires transcription and translation of the transposase gene and products, and may result in chromosomal integration later in the development of the germ-line cells. This would result in smaller numbers of transformed gametes and the resulting transformed progeny.

Southern analyses were performed on genomic DNA isolated from the transformed families and the control untransformed recipient kh^w/kh^w strain (Fig. 1). *Sac*I restriction endonuclease digestion of genomic DNA from a transformed family is expected to generate a 2.5 kb fragment consisting of a portion of the *D. melanogaster* genomic DNA encoding the cn^+ marker gene and a portion of the *Mos1* "right-hand" DNA. An additional fragment greater than 3.0 kb is generated from the *Sac*I site internal to the *D. melanogaster* DNA, the *Mos1* "left-hand" DNA, and a variable amount of mosquito genomic DNA depending on the location of the most proximal *Sac*I site. Hybridization of a radioactively-labeled probe consisting of the entire 4.7 kb *D. melanogaster* DNA should reveal these fragments. Analysis of families 52 and 52.2 clearly reveals this pattern. Family 52 shows the expected 2.5 kb internal fragment as well as another, high molecular weight fragment at ~6.1 kb characteristic of the specific insertion site. Family 52.2 has the 2.5 kb fragment and a ~6.7 kb fragment. These data are consistent with the genetic data and confirm that the original family 52 had two independent *Mos1* insertions. IPCR amplification and sequence analysis of the junction fragments of lines 52 and 52.2 indicated that both insertions are accompanied by integrations of portions of the original donor plasmid (data not shown). Donor plasmid DNA between the *Sau3AI* sites flanking both the left- and right-hand inverted terminal repeats was integrated into the mosquito genome.

Southern blot analyses with DNA isolated from the other families also showed evidence of non-canonical transposition events. For example, the hybridization patterns of families 53, P1, P3 and P12 suggest that DNA from the donor plasmid also was integrated along with the marker gene and *Mos1* DNA. Integration of plasmid DNA would yield the common 3.2 kb fragment seen along with the 2.5 kb fragment in all of the digestion patterns. Despite the similarities in the fragment sizes among the families, we are certain that each of these families represents an independent insertion because they result from different experiments or originate from different G_0 families or pools. The results of test-crosses to the parental mutant strain confirm this for two of these families and show that the insertions in families 53 and P3 are sex-linked, whereas the insertion in the P1 family is not linked to the sex locus (data not shown).

The hybridization pattern of family 23 indicates that integration of the *Mos1* DNA and marker gene were

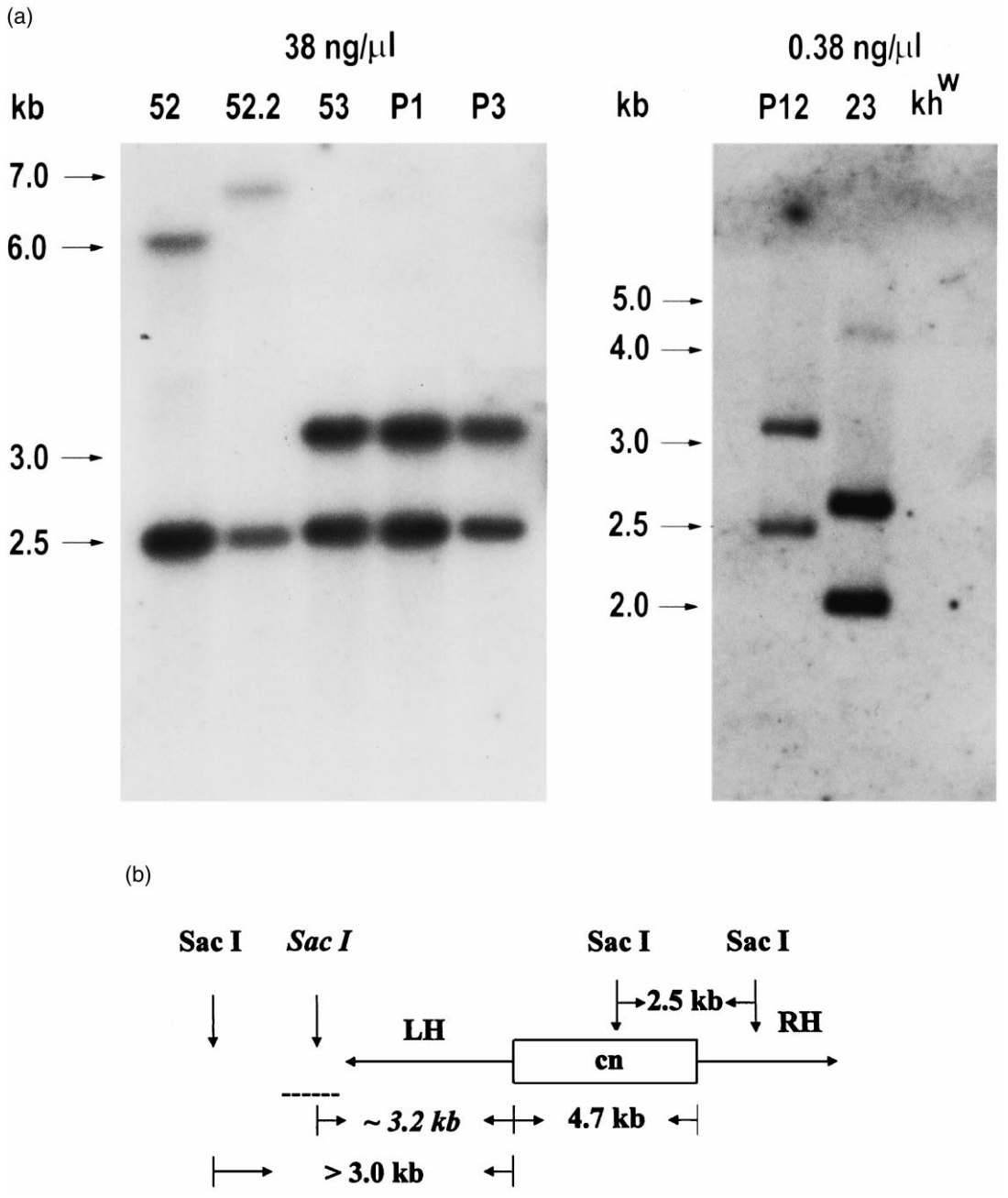

Fig. 1. Southern analyses of *Sac*I-digested genomic DNA from *Mos1* transformed families of *Ae. aegypti*. (a) Hybridization patterns observed in families generated from microinjection of 38 and 0.38 ng/μl transposase. Families 52, 52.2, 53, P1, P3 and P12 all show the 2.5 kb internal fragment. Families 52 and 52.2 show an additional high molecular-weight band at 6.1 and 6.7 kb, respectively. Families 53, P1, P3 and P12 show a 3.2 kb fragment consistent with integration of adjoining plasmid DNA. Family 23 shows three fragments, none of which are consistent with precise integration into the mosquito genome. The locations and sizes (kb) of molecular weight size markers are shown at the left of each Southern blot. (b) Schematic representation of a portion of the pM[*cn*] donor plasmid. The boxed area represents the 4.7 kb *D. melanogaster* genomic DNA fragment containing the wild-type copy of the cinnabar gene (*cn*) that was used to probe the Southern blots. The *Sac*I restriction endonuclease sites and predicted fragment sizes are shown, with those sites and fragment sizes expected from incorporation of donor plasmid indicated in italics. The dashed line represents the adjoining donor DNA that includes a *Sac*I site. The *Mos1* right-hand and left-hand terminal inverted repeats, RH and LH, respectively, are drawn as thin horizontal lines with an arrowhead showing the orientation of the fragments.

accompanied by a deletion. Three fragments are produced, 2.0, 2.6 and 4.7 kb, none of which correspond to any expected sizes. It is possible that this family has multiple insertions, as evidenced by the strongly hybridizing fragments at 2.0 and 2.6 kb. The low intensity of the 4.7 kb signal may indicate that it is a terminal fragment of an array of adjacent, multiple insertions. Multiple insertions were postulated for one of the transformants, family 128, in the original series of *Mos1* experiments (Coates et al., 1998).

Although the present numbers are small, the exclusive recovery of transformed mosquito families exhibiting non-canonical *mariner* transposition events in these experiments (7/7) stands in marked contrast to the number obtained with helper plasmid in previous studies (1/4) [only 4/5 transformed lines were available for molecular analysis in Coates et al. (1998)]. Plasmid-to-plasmid mobility assays and previous genomic integrations have shown that *Mos1* is capable of precise cut-and-paste transposition in the mosquito embryo (Coates et al., 1998). Interestingly, the *Hermes* transposable element also integrates some plasmid DNA during transposition in *Ae. aegypti* (Jasinskiene et al., 2000). It is our hypothesis that the unusual *Mos1* integrations are dependent on the presence of transposase, but in these particular cases it may be acting as a general recombinase rather than a specific transposase, or that it is catalyzing an aberrant replicative transposition event. The recovery of these types of insertions may reflect the elevated transposase levels provided by direct injection of transposase. However, at similarly high concentrations of transposase in vitro, only the expected precise *Mos1* transposition events were observed (Tosi and Beverley, 2000). Future studies may address the cause of this difference between *Mos1* transposition in vivo and in vitro. Nonetheless, for many experimental purposes the exact nature and or mechanism of integration is less important than successful integration of the introduced construct into the germ-line. Thus, the elevated frequency of transformation obtained in the presence of transposase makes *Mos1* an excellent vector for the insertion of exogenous DNA into *Ae. aegypti*.

Studies with other transposons have indicated that high levels of transposase expression can be inhibitory (Hartl et al., 1997). For example, the *Himar1* transposase shows maximal activity in vitro at 10 nM but transposition declines substantially at higher concentrations (Lampe et al., 1996). In contrast, the purified *Mos1* transposase does not show this phenomenon in vitro, instead showing maximal activity at 100 nM and higher concentrations (Tosi and Beverley, 2000). The M_r of the recombinant transposase is approximately 40,000 Da. Factoring in the 3:1 dilution of the transposase prior to injection, as well as with a 1:10 dilution expected after injection (we microinject ~10% of the egg volume for each animal), we estimated that the final concentration of transposase in the embryos was 94 nM in the experiment using the 38 ng/μl mix. This concentration is close to that observed for maximum *Mos1* transposase activity in vitro. Therefore for *Mos1*, it seems appropriate to design experiments with transposase concentrations as close to 100 nm as is possible. However, the transposase-dependent activity profiles suggest that careful consideration must be given to the level and timing of transposase expression.

The efficacy of *Mos1* for catalyzing integration stands in contrast to results obtained previously using injection of purified transposases. Purified *P* transposase can be used to transform *D. melanogaster* (Kaufman and Rio, 1991), however, the frequency of transformation at the optimum concentration of transposase was no better than what could be achieved using a helper plasmid. Similarly, *Mos1* was used to transform the domestic chicken (Sherman et al., 1998), but no effect of the addition of purified transposase along with the helper plasmid was observed.

In summary, our results show that purified *Mos1* transposase leads to an increased recovery of germ-line transformants in *Ae. aegypti*. The use of purified *Mos1* transposase may also be beneficial for the generation of transgenic families in other insect species, particularly in those cases where the conditions necessary to induce transposase expression from helper plasmids lead to high mortality in injected G_0 animals.

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