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Rapid Communication

The *piggyBac* element is capable of precise excision and transposition in cells and embryos of the mosquito, *Anopheles gambiae*

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Abstract

The *piggyBac* transposable element was tested for transposition activity in plasmid-based excision and inter-plasmid transposition assays to determine if this element would function in *Anopheles gambiae* cells and embryos. In the Mos55 cell line, precise excision of the *piggyBac* element was observed only in the presence of a helper plasmid. Excision occurred at a rate of 1 event per 1000 donor plasmids screened. Precise excision of the *piggyBac* element was also observed in injected *An. gambiae* embryos, but at a lower rate of 1 excision per 5000 donor plasmids. Transposition of the marked *piggyBac* element into a target plasmid occurred in *An. gambiae* cells at a rate of 1 transposition event per 24,000 donor plasmids. The *piggyBac* element transposed in a precise manner, with the TTAA target site being duplicated upon insertion, in 56% of transpositions observed, and only in the presence of the *piggyBac* helper. The remaining transpositions resulted in a deletion of target sequence, a novel observation for the phenomenon of *piggyBac* element insertion. 'Hot spots' for insertion into the target plasmid were observed, with 25 of 34 events involving one particular site. These results are the first demonstration of the precise mobility of *piggyBac* in this malaria vector and suggest that the lepidopteran *piggyBac* transposon is a candidate element for germline transformation of anopheline mosquitoes. Published by Elsevier Science Ltd.

Keywords: *Anopheles gambiae*; *piggyBac*; Mos55; Transposable elements; Transposition assay; Transformation; Mosquitoes

1. Introduction

The use of transposable elements to introduce foreign DNA into the genome of insect vectors, and thereby render them incompetent to transmit pathogens, is one of a number of strategies aimed at controlling the spread of human disease. With the exception of one illegitimate recombination event (Miller et al., 1987), attempts to genetically transform the African malaria vector, *Anopheles gambiae*, have been unsuccessful. However, the need to genetically manipulate this vector is great, due to the high proportion of morbidity and mortality for which it is responsible. The *piggyBac* element has been useful for transformation of several insects. It was first isolated from a *Trichoplusia ni* cell line where it caused a mutant plaque phenotype upon insertion into a baculovirus genome (Cary et al., 1989). *piggyBac* is a class II transposon, working through a DNA intermediate. The element is 2476 bp in length, encodes a single open reading frame, and terminates with 13 bp perfect inverted repeats. *piggyBac* inserts at the tetranucleotide TTAA which is duplicated upon insertion (Wang and Fraser, 1993). Unique among class II transposons is that, upon excision, the target insertion sites are left intact, leaving no footprint behind (Fraser et al., 1996). The *piggyBac* element has shown mobility in embryos of a lepidopteran, the pink bollworm (Thibault et al., 1999), and has been used to transform the dipterans *Ceratitis capitata* (Handler et al., 1998) and *Drosophila melanogaster* (Handler and Harrell, 1999). The class II transposons *Hermes* and *mariner* have been used to genetically trans-

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form the yellow fever mosquito, *Aedes aegypti* (Coates et al., 1998; Jasinskiene et al., 1998), but neither of these elements have been reported to transpose in the *An. gambiae* germline. The *Hermes* element was capable of transforming Mos55 cells (Zhao and Eggleston, 1998), but movement of the element was imprecise, similar to the mobility of *Hermes* observed in *Ae. aegypti* transformants (Jasinskiene et al., 1998). Given the demonstration of the utility of green fluorescent protein as an insect marker (Handler and Harrell, 1999; Pinkerton et al., 2000), the greatest remaining challenge to achieving germline transformation is that of finding a reliable and efficient means to introduce DNA into the genome. To quickly assess the feasibility of using one particular transposable element over another, we first determined the mobility rate for *piggyBac* without germline transformation. We used an *An. gambiae* cell line to test the mobility of the *piggyBac* element due to its amenability to screening a large number of donor plasmids at one time. We also tested the mobility of candidate *piggyBac* vectors in injected embryos. Here we show that *piggyBac* is mobile in *An. gambiae* and is a prominent candidate for germline transformation of this mosquito.

2. Materials and methods

The Mos55 *An. gambiae* cell line (Marhoul and Pudney, 1972) was used for all transfection experiments. Cells were maintained on L-15 media (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (pen/strep) at 100 U/ml and 100 mg/ml, respectively.

The DNA plasmids pB[KOα], pGDV1, and pB/hsΔsst used for the excision and transposition assays are described in Thibault et al. (1999). pB[KOα] is a *piggyBac* element vector containing a kanamycin resistance gene (kan^r), an *Escherichia coli* origin of replication (ori), and the Lac Z α-peptide inserted within the element. pB/hsΔsst is a *piggyBac* element helper plasmid with the *D. melanogaster* heat-shock (*hsp70*) promoter driving expression of the *piggyBac* transposase. This plasmid carries one intact inverted repeat and the transposase open reading frame (ORF) in an ampicillin resistant (amp^r) plasmid. pKB/hsΔsst is a kan^r version of the *piggyBac* heat-shock helper pB/hsΔsst; the 3.2 kb *Eco* RI/*Hind* III fragment from pB/hsΔsst was cut out and cloned into the *Eco* RI/*Hind* III sites of a kan^r puc19 vector (pK19, Pridmore, 1987). pGDV1 is a target plasmid that contains a *Bacillus subtilis* ori and a gene for chloramphenicol resistance (cam^r). The plasmid pK[BIGα] (constructed by G.L. Grossman) was developed as a general *piggyBac* transformation vector containing an insertion of the EGFP (Clontech) gene under the control of the baculovirus *hr5-ie1* promoter from plasmid pIE1-SV-EGFP (Huynh and Zieler, 1999), and the Lac Z coding sequence, in a kanamycin-resistant vector (Fig. 1). The *piggyBac* plasmid-based excision and transposition assays were conducted essentially as in Thibault et al. (1999).

Mosquito cell transfection experiments were carried out in the following manner: Mos55 cells were grown at 28°C in 25-cm flasks to 80% confluency, and immediately before transfections, rinsed twice with 5 ml of L-15 media in the absence of FBS and pen/strep. The lipid transfectant Cellfectin (Gibco/BRL) was prepared at 10 μg/ml with plasmid DNA (at a final concentration of 5 μg/ml) in L-15 media. Lipid/DNA mixtures were allowed to form complexes for 45 min at RT (room temperature), according to the manufacturer's instructions. Amounts of DNA for each experiment were as follows: for the excision assays, 2 μg/ml pB/hsΔsst and 3 μg/ml pB[KOα]; for excision controls, 5 μg/ml pB[KOα]; for the transposition assays, 2 μg/ml pB[KOα], 2 μg/ml pGDV1, and 1 μg/ml pB/hsΔsst; for transposition controls, 2.5 μg/ml pB[KOα] and 2.5 μg/ml pGDV1. Two ml of lipid/DNA mixture was applied to cells and incubated for 20 h. Transfection mixes were then removed and normal growth medium was applied to the cell monolayer. Twenty hours later, cells were washed twice with 5 ml of 1X PBS (phosphate-buffered saline; Mg^{2+}- and Ca^{2+}-free), then 1 ml Hirt lysis buffer (Hirt, 1967) was applied and incubated 10 min at RT to recover plasmid DNA.

Mosquitoes were cultured by standard methods (Benedict, 1997) and embryos were collected and injected essentially as in Morris (1997). For excision assays in embryos, 0.5 μg/ml pB[KOα] and 0.2 μg/ml pB/hsΔsst in PBS were injected; for helperless excision controls, 0.5 μg/ml pB[KOα] alone were injected. Injected embryos were incubated for 24 h at 27°C. After incubation, embryos were homogenized and plasmid DNA was recovered as described below.

Low-molecular-weight plasmid DNA was isolated from cells and homogenized embryos following the procedure of Hirt (1967). Recovered plasmids were screened and analyzed for excision or transposition events, essentially as in Sarkar et al. (1997a,b). Purified plasmid DNA was electroporated into DH10B electroMAX *E. coli* cells (Gibco/BRL) using a Cellporator and Voltage Booster (BRL) at 400 V, 4 kΩ, and 330 μF capacitance. Transformed cells were plated on selective media and the number of colonies counted to determine the original number of donor plasmids transfected into Mos55 cells. DNA from colonies representing potential excision and transposition events was isolated using the Wizard Miniprep kit (Promega). Restriction enzyme analyses identified those colonies most likely to represent real excision and transposition events. Junctions of these were then sequenced on an ABI 377 automated sequencer (Perkin Elmer/Applied Biosystems).

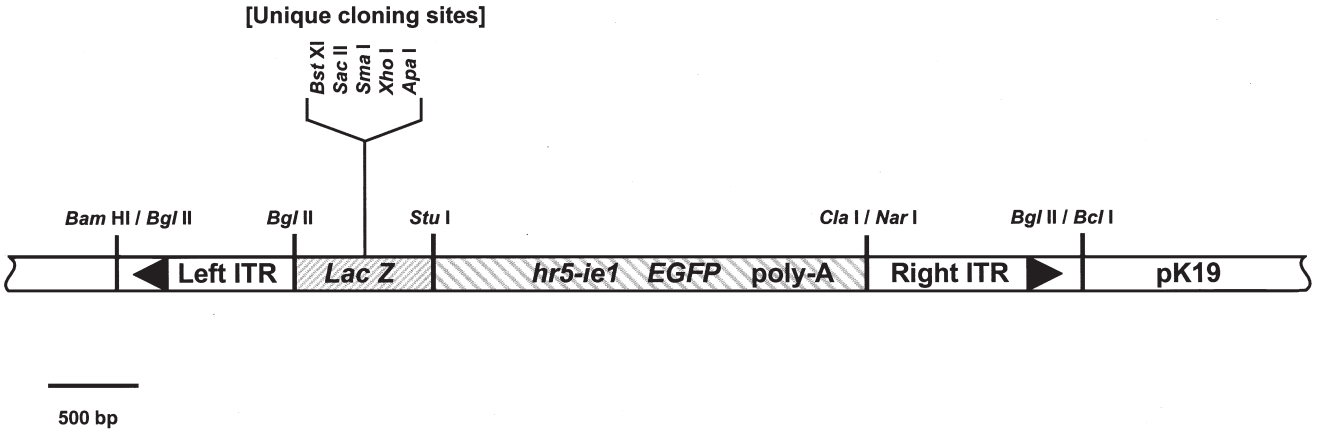

Fig. 1. Restriction map of pK[BIGα], a *piggyBac* transformation vector. pK[BIGα] was constructed in the following manner: the 3 kb *Bgl* II [KOα] fragment was removed from pB[KOα] (resulting in the plasmid pB[-KOα]), leaving a unique *Bgl* II site between the *piggyBac* ITRs; a 2.8 kb *hr5-ie1*/*EGFP*/poly-A cassette was PCR amplified from plasmid pIE1-SV-EGFP (Huynh and Zieler, 1999) using primers that contained *Nar* I and *Stu* I sites; a 700 bp *Lac Z* α-peptide gene was PCR amplified with *Stu* I and *Bgl* II ends; the *hr5-iel*/*EGFP* and *Lac Z* fragments were simultaneously cloned into the *Bgl* II/*Cla* I sites of plasmid pB[-KOα]. The 5450 bp *piggyBac*/EGFP/LacZ insert was removed by digestion with *Bcl* I and *Bam* HI, then ligated into a unique *Bgl* II site in pK19 which had been generated through site-directed mutagenesis (M.Q. Benedict, unpublished). The endogenous *Lac Z* gene in pK19 had been deleted prior to this cloning. The resulting plasmid, pK[BIGα], is 7370 bp and kanamycin resistant. Blue/white selection for cloned inserts is possible at the unique cloning sites shown. It is also possible to use the unique sites *Bgl* II and *Stu* I in conjunction with one of the unique restriction enzyme sites within the *Lac Z* gene. Sequence information may be obtained from the corresponding author.

3. Results and discussion

Results from all of the excision experiments with *piggyBac* are listed in Table 1. We found that *piggyBac* excised at a frequency of 0.1% in the Mos55 cell line. In contrast, when *piggyBac* plasmids were injected into *An. gambiae* embryos, the excision frequency decreased five-fold to 0.02%. In one experiment conducted to test the EGFP-marked *piggyBac* transformation vector, pK[BIGα], we observed five excision events out of 469 donors screened (1.07%). No excisions were observed in the absence of a helper plasmid source for *piggyBac* nor were any excision events observed when the *piggyBac* plasmids were electroporated directly into the DH10B *E. coli* cells. This confirmed that the *piggyBac* excision events we observed took place within the mosquito embryos and Mos55 cells.

After determining the excision frequency of *piggyBac* in cells and embryos, we tested the ability of this element to transpose into a target plasmid in cells. In five experiments that tested *piggyBac* transposition, we observed consistent results which showed a transposition frequency of 0.0042% (Table 2) or 1 event per 24,000 donor plasmids screened. The *Hermes* element in injected *Ae. aegypti* embryos transposed at a rate of 0.00286%, or 1 event per 35,000 donor plasmids (Sarkar et al., 1997b). Additionally, the transposition rate for the *mariner* element in *Ae. aegypti* was reported to be 0.0009% (Coates et al., 1998). Even if our transposition results for *piggyBac* in the *An. gambiae* cell line were five-fold lower in embryos—as is the case for *piggyBac* excision in both of these systems tested—the predicted transposition rate of 0.00084% is still similar to that observed for *mariner* in *Ae. aegypti*. Since both *mariner*

Table 1
piggyBac excision events recovered from *An. gambiae* cells, injected embryos, and controls

Recipient	Donor	Helper	No. exps.	No. donors	No. excisions	% excision[a]
Cells	pB[KOα]	pKB/hsΔsst	3	2.2×10^7	2004[b]	0.1
Cells	pB[KOα]	None	3	$>4\times10^6$	0	0
Cells	pK[BIGα]	pB/hsΔsst	1	469	5	1.07
Cells	pK[BIGα]	None	1	105	0	0
Embryos	pB[KOα]	pKB/hsΔsst	2	1.7×10^5	9	0.02
Embryos	pB[KOα]	None	2	4×10^5	0	0
E. coli	pB[KOα]	pKB/hsΔsst	1	$>1\times10^6$	0	0
E. coli	pB[KOα]	None	1	$>1\times10^6$	0	0

[a] Excision frequency was calculated for each experiment and then averaged over all experiments.
[b] The total number of excisions was estimated from screening 20% of white colonies obtained.

Table 2
Transposition of piggyBac in *An. gambiae* cells and controls

Recipient	Donor	Target	Helper	No. exps.	No. donors	% transposition[a]
Cells	pB[KOα]	pGDV1	PB/hsΔsst	5	4.5×10^6	0.0042
Cells	pB[KOα]	pGDV1	None	4	2.1×10^6	0
E. coli	pB[KOα]	pGDV1	pB/hsΔsst	1	$>1\times10^6$	0
E. coli	pB[KOα]	pGDV1	None	1	$>1\times10^6$	0

[a] Transposition frequency was calculated for each experiment and then averaged over all experiments.

and *Hermes* have subsequently been used as successful transformation vectors in *Ae. aegypti* (Coates et al., 1998; Jasinskiene et al., 1998), it is reasonable to predict that the *piggyBac* element should be a useful element to use for *An. gambiae* transformation experiments.

A total of 34 *piggyBac* transposition plasmids isolated from *An. gambiae* cells were sequenced at both ends of the insertion site. All but one of the insertions occurred at a TTAA target site, which was duplicated upon insertion (Table 3). Of those, 19 (56%) were precise transposition events, but 15 transpositions resulted in a partial deletion of target plasmid sequence. One of the latter class was the exceptional insertion in which the right ITR inserted at a GAAA (nt 641) whereas the left ITR inserted at a TTAA (nt 993). These deletions may have been due to an overabundance of transposase enzyme present in the cells that resulted in nicking and/or multiple insertions and excisions. Alternatively, this phenomenon may have occurred as a result of multiple insertion events into one target plasmid followed by recombination between inserted elements. We were unable to obtain sequence at one of the two ends from one additional transposition plasmid, possibly due to loss of the primer sites. Among the 34 transpositions sequenced, 25 had at least one end of the element inserted at position 993 in the target plasmid. Overall, there were only 8 positions in pGDV1 in which *piggyBac* inserted. Insertion was therefore not a random event, but specifically mediated by the *piggyBac* transposase. We observed no insertions of *piggyBac* in the sequence downstream from the chloramphenicol resistance gene, unlike Thibault et al. (1999) who observed four insertions in this region, three of which were also insertion sites for the *mariner* element in transposition assays performed in *Ae. aegypti* embryos (Coates et al., 1998).

Table 3
Sites of precise piggyBac insertion in pGDV1 in transfected Mos55 cells

	Left ITR insertion site	Base[a]	Insertion orientation[b]	Base	Right ITR insertion site	No. events
Precise insertions	GAAATCGACT**TTAA**	993	+	994	**TTAA**AAGAGAATGA	11
	GAAATTGAGA**TTAA**	102	+	103	**TTAA**GGAGTCGATT	**2**
	CAAACAAAGTTTAA	**364**	+	**365**	**TTAA**AGCTAAAGCA	**2**
	AAGCAATTGATTAA	**946**	+	**947**	**TTAA**AGAAATATCT	**2**
	GAGCGATTCCTTAA	**86**	+	**87**	**TTAA**ACGAAATTGA	**1**
	AATGCTTTGGTTAA	**492**	+	**493**	**TTAA**AACAAAATAT	**1**
Insertions with target deletions	**GAAATCGACTTTAA**	**993**	**−**	**87**	**TTAA**ACGAAATTGA	**4**
	GAGCGATTCCTTAA	**86**	+	**994**	**TTAA**AAGAGAATGA	**2**
	TCCAATTGATTTAA[c]	**969**	+	**994**	**TTAA**AAGAGAATGA	**2**
	GAAATCGACTTTAA	**993**	**−**	**970**	**TTAA**CGGAATTAAT	**2**
	GAGCGATTCCTTAA	**86**	+	**103**	**TTAA**GGAGTCGATT	**1**
	AAGCAATTGATTAA	**946**	+	**994**	**TTAA**AAGAGAATGA	**1**
	GAAATCGACTTTAA	**993**	**−**	**641**	**TTAA**AACAAAATAT[d]	**1**
	AATGCTTTGGTTAA	**492**	+	**994**	**TTAA**AAGAGAATGA	**1**
	GAAATTGAGATTAA	**102**	+	**994**	**TTAA**AAGAGAATGA	**1**
	GAAAT-GACTTTAA		**>60% Consensus**[e]		**TTAA**A-GAAA-TGA	

[a] Numbers indicate basepair positions in target plasmid pGDV1.

[b] Orientation of *piggyBac* insertions were in the + orientation when the ORF was 5′–3′ with respect to the chloramphenicol-resistance gene coding sequence in pGDV1.

[c] Unusual bases that differ greatly from the calculated consensus are clustered in this insertion site (underlined and bold).

[d] Exceptional junction junction where the right ITR of *piggyBac* did not insert at a TTAA site in pGDV1, although the consensus TTAA sequence was present at the 3′ end of the ITR.

[e] Calculated on base appearing at least 60% in a position when frequency of insertion site is considered, i.e. weighted.

It remains unclear whether there is a defined consensus insertion site beyond the TTAA sequence. In *piggyBac* mutagenesis experiments of baculovirus, Cary et al. (1989) concluded that there was an extended consensus consisting of YYTTTTTT/AARTAAYAG (Y=pyrimidine, R=purine, /=insertion site). Our insertions into pGDV1 also show an extended consensus (Table 3) which, although it is generally weak, at several positions is in accord with that of Cary. In contrast, neither Thibault et al. (1999) studying interplasmid transposition in lepidopteran embryos nor Handler et al. (1998) who studied *D. melanogaster* genomic insertion sites identified a consensus beyond the TTAA except that the region tends to be AT rich. One site into which we observed two independent insertions (at nt 969) contained a large number of bases that were especially inconsistent with the general consensus (underlined bases in Table 3). Considering the different hosts and targets in these experiments, it is difficult to make firm conclusions regarding an extended consensus. In our experiments, certain bases do occur with high frequency at several positions. For example, there are four guanines located at positions 1, 7, 17, and 23 in the illustrated consensus surrounding the target insertion site (Table 3). This observation suggests that the preferred target site region has sequence characteristics beyond simply being AT rich.

In addition to the strict target site preference, the *piggyBac* element showed strong insertion orientation preference in the target plasmid depending on whether the insertion was precise or resulted in a deletion. All 19 precise events were in the same orientation (5′–3′, with respect to the chloramphenicol resistance gene open reading frame); however, eight of 15 deletion events resulted in *piggyBac* in the opposite orientation. In other words, even though precise events were uniformly oriented, the deletion events were equally distributed between the two orientations. We offer two explanations for why this might be. While it is possible that the *piggyBac* element inhibits plasmid replication or selectable marker expression in *E. coli* by any of several means when in the reverse orientation unless regions of pGDV1 are deleted, we find this explanation rather unlikely. Moreover, this explanation suggests nothing about the mechanism of transposition or events leading to the deletions. It seems more likely that recombination events within the element, perhaps between the ITRs and/or the internal inverted repeats of one or more elements in the same target plasmid, result in occasional inversion of the element and concomitant deletion of intervening DNA. No other reports on *piggyBac* mobility assays have described this phenomenon of target sequence deletion.

In our hands, the *Hermes* element does not appear as attractive as *piggyBac* as a transformation vector in *An. gambiae*. In assays similar to those described above, we have observed only extremely limited mobility of *Hermes* (0.001% excision in three Mos55 cell experiments, G.L. Grossman, unpublished). Also the presence of an endogenous hAT-like element, *huni*, in *An. gambiae* (G.L. Grossman, unpublished) could be capable of trans-activation of a *Hermes* transformation element, although no *Hermes* mobility has been observed in the absence of helper plasmid thus far. Zhao and Eggleston (1998) were able to transform an *An. gambiae* cell line using *Hermes*, but they did not perform any plasmid-based excision or transposition assays with this element, therefore no direct comparison may be made with our *piggyBac* mobility data.

With endogenous *Hermes* elements present in *An. gambiae*, uncontrolled movement of the element within or out of the genome could result. Future considerations and concerns regarding containment of introduced transposable elements need to address the possibility of movement of these elements when endogenous copies exist in the genome of the organism being tested. These facts were taken into consideration when deciding to focus on and develop the *piggyBac* element as a transformation vector for *An. gambiae*. No excision or transposition of *piggyBac* has been observed in *An. gambiae* in the absence of a helper plasmid. Neither is there is any indication of the presence of endogenous *piggyBac* elements in the genomes of *An. gambiae*, *An. quadrimaculatus*, *An. dirus*, *An. stephensi*, *An. albimanus*, or *Ae. albopictus*, as tested by PCR and Southern blot analysis (data not shown).

Experiments similar to those we have performed have been done in *An. gambiae* cell lines and embryos using the *Minos* element. Catteruccia et al. (2000) demonstrated that *Minos* is capable of transposase-mediated excision and insertion into the chromosomes of *An. gambiae* cell lines. Furthermore, they showed that interplasmid transposition of *Minos* in *An. stephensi* occurred at a rate of 0.013% in *An. stephensi* embryos. It is impossible to compare their data with ours, however, as their interplasmid experiments were all performed in injected embryos. Furthermore, although they reported excision activity in cells, no quantitative data were presented for comparison purposes. They did clearly demonstrate that the *Minos* element is another worthwhile candidate for *Anopheles* transformation.

All of the essential elements for transformation of *An. gambiae* appear to be functional insofar as they can be tested without transformation itself. In support of this conclusion, we have learned that, very recently, the *piggyBac* element was successfully used to transform the mosquito *An. albimanus* (A. Handler, personal communication). We are currently injecting pK[BIGα] into *An. gambiae* embryos and screening the G_1 progeny for EGFP expression.

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