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Post-Integration stability of *piggyBac* in *Aedes aegypti*

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Abstract

The post-integration activity of *piggyBac* transposable element gene vectors in *Aedes aegypti* mosquitoes was tested under a variety of conditions. The embryos from five independent transgenic lines of *Aedes aegypti*, each with a single integrated non-autonomous *piggyBac* transposable element gene vector, were injected with plasmids containing the *piggyBac* transposase open-reading frame under the regulatory control of the *Drosophila melanogaster* *hsp70* promoter. No evidence for somatic remobilization was detected in the subsequent adults whereas somatic remobilization was readily detected when similar lines of transgenic *Drosophila melanogaster* were injected with the same *piggyBac* transposase-expressing plasmid. *Aedes aegypti* heterozygotes of *piggyBac* reporter-containing transgenes and *piggyBac* transposase-expressing transgenes showed no evidence of somatic and germ-line remobilization based on phenotypic and molecular detection methods. The post-integration mobility properties of *piggyBac* in *Aedes aegypti* enhance the utility of this gene vector for certain applications, particularly those where any level of vector remobilization is unacceptable.

Keywords

piggyBac; *Aedes aegypti*; *Drosophila melanogaster*; transposable elements; mosquitoes

Introduction

The gene vectors currently used for non-Drosophilid insect germ-line transformation are class II transposable elements with short terminal inverted repeats that move via a DNA-mediated intermediate using a cut-and-past mechanism (Atkinson et al., 2001). The availability of broad host range transformation systems constructed from the insect transposable elements *Hermes*, *Mos I*, *Minos*, and *piggyBac* provide researchers with tools both for functional genomic studies and the development of novel insect biological control applications (Handler and James, 2000). The suitability of gene vectors for particular applications is based in part on their post-integration behavior in insect genomes (O'Brochta et al., 2003). For instance, transposon mutagenesis, enhancer trapping, and the use of transposable element-based gene vectors as gene spreading genetic drive systems in insects will require transposable elements with high rates of remobilization in the presence of transposase. For functional genomics studies and using transgenic insects as bioreactors transgene stability within the genome will be essential. Transgene instability in the form of

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vector excision or transposition could, in this case, compromise the integrity of the transformed strain and severely limit its utility. To date, most studies of insect gene vectors have focused on their functionality in particular species and few have examined the post-integration behavior of the elements. Sundararajan et al. (1999) found that non-autonomous *Hermes* elements integrated in the genome of *D. melanogaster* were unstable as a consequence of cross mobilization resulting from the expression of transposase from native *hobo* elements (Sundararajan et al., 1999). The *mariner* element *Mos 1* when integrated into the genome of *Aedes aegypti* was found to rarely remobilize in the presence of functional *Mos 1* transposase (Wilson et al., 2003), a behavior that was similar to that reported in *Mos 1*-containing transgenic *D. melanogaster* (Lozovsky et al., 2002).

The Lepidopteran transposable element *piggyBac* (Cary et al., 1989; Fraser et al., 1995) has been widely used for germ-line transformation in phylogenetically diverse organisms including protozoa, planaria, insects and mammals (Balu et al., 2005; Ding et al., 2005; Gonzalez-Estevéz et al., 2003; Handler, 2002). Insects transformed with *piggyBac* include those in the orders Lepidoptera (Marcus et al., 2004; Peloquin et al., 2000; Tamura et al., 2000), Hymenoptera (Sumitani et al., 2003), Coleoptera (Kuwayama et al., 2006; Lorenzen et al., 2003) and Diptera (Grossman et al., 2001; Handler and Harrell, 2001; Handler et al., 1998; Hediger et al., 2001; Kokoza et al., 2001; Li et al., 2001; Nolan et al., 2002; Perera et al., 2002). However, few studies on the post-integration behavior of the *piggyBac* elements have been reported (Horn et al., 2003; Lorenzen et al., 2003). In *D. melanogaster* *piggyBac* remobilized readily in the presence of *piggyBac* transposase and displayed mobility rates comparable to the widely used *P* element gene vector in this species (Horn et al., 2003). Integrated *piggyBac* elements could also be remobilized in the beetle *Tribolium* and, while not as thoroughly studied as in *D. melanogaster*, remobilization appeared to be common in the presence of *piggyBac* transposase (Lorenzen et al., 2007).

Preliminary work on *piggyBac* remobilization in *Ae. aegypti* revealed the absence of any post-integration remobilization of *piggyBac* either in the germ-line or soma (O'Brochta et al., 2003). Here we report on a more comprehensive study of the stability of *piggyBac* in *Ae. aegypti* in both the presence and absence of functional transposase and found it to be, under all conditions tested, highly stable.

Materials and Methods

Insect strains

All *Ae. aegypti* strains were reared under standard laboratory conditions as described (Wilson et al., 2003). Transgenic lines created specifically for this study were based on the *kh^w* strain (Bhalla, 1968). Lines MF1.92 and MF03 have been described and are transgenic lines also based on the *kh^w* strain (Lobo et al., 2002). The line PE11 was based on the wild-type laboratory strain 'Bangkok' (Nimmo et al., 2006).

Drosophila melanogaster lines were obtained from the Bloomington Drosophila Stock Center at Indiana University and maintained at 25°C except where noted. The following lines were used: 17861 = *w¹¹¹⁸*; *PBac{RB}CG6347^{e00490}*; 17975 = *w¹¹¹⁸*; *PBac{RB}CG17600^{e01638}* *CG17598^{e01638}*; 18050 = *w¹¹¹⁸* *PBac{RB}CG2247^{e02382}*; 18256 = *PBac{RB}CG4199^{e04396}* *CG4061^{e04396}* *w¹¹¹⁸*; 18759 = *w¹¹¹⁸*; *PBac{WH}CG13131^{f04310}*; 8285 = *w¹¹¹⁸*; *CyO*, *P{Tub-PBac|T}2/wg^{Sp-1}*

Vector Constructions

pBac::hspDsRed::cn—This 'reporter' element-containing plasmid was constructed using functional *piggyBac* inverted repeats containing approximately 0.7 kb of sub-terminal

sequences of the right and left ends of *piggyBac*, a 4.7 kb genomic DNA fragment from *D. melanogaster* containing the *cinnabar* (*cn*) gene and *DsRed* containing a minimal promoter that would make it subsequently useful as an enhancer-detector and potentially useful in detecting remobilizations. The left and right end of *piggyBac* were re-ligated after cutting the plasmid *pBac3xP3EGFP-Mos* (Wilson et al., 2003) with *Pst* I to remove the EGFP marker gene and creating the plasmid *pBac700RL*. The 1kb *Xho*I/*Not*I fragment containing *DsRed* and the minimal promoter from *pDsRed* (Clontech) was excised and inserted into the universal shuttle vector *pSLfa1180fa* to create the plasmid *pSL1180::hspDsRed* (Horn and Wimmer, 2000). A DNA fragment containing the *cn* gene was isolated from *pBCKScn* following digestion with *Xho*I and inserted into the *Xho*I site of *pSL1180::hspDsRed* to create *pSL1180::hspDsRed::cn*. Finally, the *hspDsRed::cn* fragment was isolated following digestion with *Asc*I and inserted into the *Asc*I site of *pBac700RL* to yield the final plasmid, *pBac::hspDsRed::cn*.

pMos3xP3EGFP-pBac—This plasmid contains a *Mos* I vector with the *piggyBac* transposase open reading frame under the regulatory control of the *D. melanogaster* heat shock 70 (*hsp70*) promoter and an EGFP marker gene. The *D. melanogaster* *hsp70* promoter is known to be active in the germ-line of *Ae. aegypti*, as it has been included in ‘helper plasmids’ used to create transgenic *Ae. aegypti* (Coates et al., 1998; Jasinskiene et al., 1998; Kokoza et al., 2000; Kokoza et al., 2001; Lobo et al., 2002; Pinkerton et al., 2000; Wilson et al., 2003). The 3.5kb *piggyBac* transposase-containing *Asc*I fragment from the plasmid *pMi3XP3DsRed-hsp-pBac* (a gift from Dr. Alfred Handler, USDA, Gainesville, Florida) was inserted into the *Asc*I site of *pMos3xP3-EGFP* (Horn and Wimmer, 2000) to yield *pMos3xP3EGFP-pBac*.

pMos3xP3EGFP-pBac^G—This plasmid is identical to *pMos3xP3EGFP-pBac* except it contains a single nucleotide deletion in codon 493 resulting in a frame shift mutation. The protein predicted from this mutated transposase gene will be 502 amino acids in length, compared to the full length protein of 594 amino acids, and is not expected to be a functional transposase.

Inter-Plasmid Transposition Assay

Inter-plasmid transposition assays in *D. melanogaster* embryos were performed essentially as described except the plasmid *pMos3xP3EGFP-pBac* was used as ‘helper plasmid’ and source of functional *piggyBac* transposase (Lobo et al., 1999; Sarkar et al., 1997). All putative transposition events were analyzed by restriction mapping and confirmed by DNA sequencing.

Germ-line Transformation

Mosquito germ-line transformations using *pMos3xP3EGFP-pBac* and *pBac::hspDsRed::cn* and transgenic strain-establishment were performed as described (Wilson et al., 2003). To create *piggyBac* transposition reporter strains a mixture of *pBac::hspDsRed::cn* (0.3 µg/µl) and *pBac/hsΔsst* (0.2µg/µl) (Handler and Harrell, 1999) was injected into preblastoderm *kh^w* embryos (Bhalla, 1968). Similarly, *piggyBac* transposase-expressing helper lines were created by injecting a mixture of *pMos3xP3EGFP-pBac* and *pkhsp82Mos* into preblastoderm *kh^w* embryos. Control lines expressing a nonfunctional *piggyBac* transposase following the integration of the gene vector contained on *pMos3xP3EGFP-pBac^G* were created in a similar way. Injected embryos were heat-shocked at 41 °C for 1 hour between 16 and 24 hours after injection and reared to adulthood. Adults developing from injected embryos (G₀ adults) were separated according to sex and small families consisting of approximately five G₀ males or females were mated to approximately 10-15 *kh^w* individuals of the opposite sex. Transformed G₁ progeny were identified by their pigmented eyes in the

case of the transposition reporter lines containing *pBac::hspDsRed::cn* (lines 40D and 40L) and the presence of the expression EGFP in the eyes and brain in the case of lines arising from the vectors contained on *pMos3xP3EGFP-pBac* (lines C09,I03,I12,J02 and J26) and *pMos3xP3EGFP-pBac^G* (lines 32, 37A, 38).

The creation of *piggyBac* reporter lines MR1.92, MF03 and PE11 has been described. Lines MF 1.92 and MF03 correspond to lines I and III, respectively, described in Lobo et al. (2002). The line PE11 corresponds to Strain 11 described by Nimmo et al. (2006).

Genetic Analysis

Non-autonomous *piggyBac* strains, 40D, 40L, MR1.92, MF03 and PE11 were crossed to the *piggyBac* transposase-containing strains 32, 37A, 38 (containing *pMos3xP3EGFP-pBac^G* expressing non-functional transposase) and C09, I03, I12, J02 and J26 (containing *pMos3xP3EGFP-pBac* expressing functional transposase). The resulting heterozygotes (F1) were either heat-shocked daily as larvae for 1 hour at 41°C until pupation, or reared at a constant 28°C, and the adults were crossed in small pools of approximately ten individuals of one sex to approximately 20 *kh^w* individuals of the opposite sex. Adults emerging from this cross (F2) were screened for changes in eye color (lighter or darker than the parental eye color) two days after emergence. Adults arising from these crosses with eye colors differing from the parental eye color of heterozygous 40D, 40L, MR1.92, MF03 and PE11 were considered to be possible products of element remobilization resulting in altered reporter gene expression due to chromosomal position effects. Individuals containing putative transposition events were crossed to *kh^w* to expand the numbers of individuals with the new genotype. The red-eyed F2 parental mosquito was sacrificed for analysis by transposable element display.

Transposable Element display

Transposable element display was performed essentially as described with modifications to adapt the method to *piggyBac* elements (Guimond et al., 2003). Briefly, 50-100 ng of mosquito genomic DNA from a single insect from a *piggyBac* element-containing transgenic line was digested with *MspI* (New England Biolabs) according to the manufacturer's recommendation. Sixty pmoles of *MspI* adapters (consisting of a dimer of the oligonucleotides *MspI*a 5'-GAC GAT GAG TCC TGA G-3' and *MspI*b 5'-CGC TCA GGA CTC AT-3') were ligated to digested gDNA in the presence of *MspI* and the restriction/ligation reaction was allowed to continue overnight and then diluted 4x with 0.1x TE buffer. 'Pre-selective' PCR was performed using 5 µl of the restriction/ligation mixture as template with primers *MspI*a and piggyL1 (5'-TAT GAG TTA AAT CTT AAA AGT CAC G-3') for the left inverted terminal repeat specific display and *MspI*a and piggyR1 (5'-GTT GAA TTT ATT ATT AGT ATG TAA GTG-3') for inverted terminal repeat specific display. Reaction conditions included an initial denaturation step of 3 min at 95°C followed by 25 cycles of 15 s at 95°C, 30 s at 54°C and 1 min at 72°C with a final 5 min elongation at 72°C. The PCR products were diluted twenty times with 0.1x TE (1 mM Tris HCl, pH 7.5, 0.1mM EDTA) and 5 µl used as a template in a second PCR. This 'selective' PCR used primers *MspI*a and Cy5-labelled piggyL2Cy5 (5'-CAG TGA CAC TTA CCG CAT TAC AAG C-3') for the left inverted terminal repeat specific display and *MspI*a and Cy5-labelled piggyR2Cy5 (5'-ATA TAC AGA CCG ATA AAA ACA CAT GCG) for the right inverted terminal repeat specific display. The reaction conditions included an initial denaturation step of 3 min at 95°C, followed by five cycles of 'touchdown' PCR consisting of a denaturation step of 15 s at 95°C followed by annealing for 30 s at a temperature that was reduced by one degree on each successive cycle beginning at 59°C and an extension step of 1 min at 72°C. The touchdown phase of the reaction was followed by 25 cycles of 15 s at 95°C, 30 s at 54°C and 1 min at 72°C with a final elongation of 5 min at 72°C.

Analyses of the *Mos1* element-containing transgenic lines ("Helper" lines) were done using a similar protocol except genomic DNA was digested with *MseI*. *MseI* adaptors (consisting of a dimer of the oligonucleotides *MseI*a 5'-GAG TCC TGA GTA GCA G-3' and *MseI*b 5'-TAC TCA GGA CTC AT-3') were used during the restriction/ligation step. The 'pre-selective' PCR was run using the primers *MseI*a and MOS187r (5'-TGT CCG CGT TTG CTC TTT ATT CG-3') with reaction conditions of 3 min at 95°C followed by 25 cycles of 15 s at 95°C, 1 min at 60°C and 1 min at 72°C, finishing with 5 min at 72°C. The 'selective' PCR was performed using primers *MseI*a and the Cy5-labelled MOSCy5::46r (5'-ACA ATC GAT AAA TAT TTA CGT TTG C-3'). This reaction involved an initial 3 min denaturation at 95°C followed by five cycles of 'touchdown' PCR consisting of a denaturation step of 15 s at 95°C followed by annealing for 1 min at a temperature that was reduced by one degree on each successive cycle beginning at 64°C and an extension step of 1 min at 72°C. The 'touchdown' phase of the reaction was followed by 25 cycles of 15 s at 95°C, 1 min at 64°C, and 1 min at 72°C, finishing with an elongation step of 72°C.

PCR products were size-fractionated on a 6% poly-acrylamide gel under denaturing conditions (6M Urea) and viewed, after drying on 3 MM paper, on a Storm 860 optical scanner (Molecular Dynamics) using the excitation wavelength of 635 nm. Bands of interest were excised from the gel, re-amplified using the 'selective' PCR protocol and unlabeled primers, purified using Wizard PCR preps (Promega), and sequenced.

Transcript analysis

RNA extraction using an RNEasy kit (Qiagen) and complementary DNA synthesis from the purified RNA was essentially performed as described (Wilson et al., 2003). The resulting cDNA (1 µl) was used as a template in the following PCR in which *piggyBac* transposase cDNA was detected using the primers pBac3219f (5'-TTC AAA GTC CAC GAG GCG3') and pBac4208r (5'-GTA CTT TCGTTG ATA GAA GCA TCC-3'). The cycle conditions included an initial denaturation step of 3 min at 95°C, followed by 35 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 1 min with a final elongation step of 72°C for 5 min.

Cytoplasmic actin cDNA was detected (as a positive control) by performing PCR simultaneously under the conditions as described above except with primers Actinf (5'-TGG ACT TCG AGC AGG AAA TGC-3') and Actinr (5'-CTG GGC GCG GAA ACG-3') and the reactions were removed after 25 cycles. All PCR products were size fractionated by electrophoresis in agarose.

Methylation detection

The methylation status of the terminal sequences of the *piggyBac* element was investigated by initially digesting genomic DNA with *MspI*. The digested gDNA was size fractionated on a 2% metaphor agarose gel. DNA of the desired size was purified from the gel using Gene Clean Turbo (BIO 101). Sodium bisulfite treatment of isolated DNA fragments was performed according to the method described by Clark et al. (1994). Primers used to amplify the terminal regions of the integrated *piggyBac* elements after treatment with sodium bisulfite were: 40L Left ITR forward (5'-AAT TGA TAT AAA TTT GGA GTTTTA ATT 3'), 40L Left ITR reverse (5'-TAT AAT TCA AAA TCA ATA ACA CTT ACC A 3'), 40L Right ITR forward (5'-GTA TAT TAG ATG ATA GTA TTG AAG AGT 3'), 40L Right ITR reverse (5'-TAT AAT TCA AAA TCA ATA ACA CTT ACC A 3'). PCR conditions used here were similar to those used for PCR screening for *piggyBac* element described above. The newly amplified PCR products were cloned (TOPO TA cloning kit) and sequenced.

Results

Construction of non-autonomous piggyBac 'reporter' lines

Two lines, 40D and 40L, were created following the independent integration of the *piggyBac* vector, *pBac::hspDsRed::cn*. The presence of a single integrated element in each line was confirmed by observing the pattern of inheritance of the marker gene and transposable element display. Integrations resulted from canonical cut-and-paste transposition and resulted in the creation of TTAA direct duplications flanking the integrated elements, as was expected when using *piggyBac* vectors. The structural integrity of the *piggyBac* inverted-terminal-repeat sequences present within the integrated vectors was determined following PCR and DNA sequencing and all were found to be mutation free. Therefore, based on the structure of the integrated *piggyBac* elements in these two lines they appeared to be fully competent to undergo transposition. Three additional reporter lines created by others, MF1.92, MF03, PE11, (Lobo et al., 2002; Nimmo et al., 2006) were also used in the experiments reported here and were found to have similar characteristics.

Construction of *piggyBac* 'helper' lines

Before using the vector *pMos3xP3EGFP-pBac* to create transgenic 'helper' lines that expressed *piggyBac* transposase the ability of this vector to produce functional transposase was tested. The plasmid *pMos3xP3EGFP-pBac* was used as a 'helper plasmid' in a *piggyBac* inter-plasmid transposition assay in *D. melanogaster* embryos. In a series of two experiments, 261,000 pGDV1 target plasmids were screened resulting in 44 confirmed independent transposition events being recovered for an inter-plasmid transposition frequency of 2×10^{-4} transpositions per target plasmid screened. Transposition events were confirmed by DNA sequencing and their distribution within the target plasmid determined (Figure 1). The frequency and pattern of transposition seen here was similar to that reported by Lobo et al. (2001) and Grossman et al. (2000). In all cases integrations were into TTAA target sites and the target sites used comprised the same subset of target sites observed to be preferred *piggyBac* integrations sites by Grossman et al. (2000) indicating that the *hsp::piggyBac* transposase transgene in *pMos3xP3EGFP-pBac* is fully functional and capable of producing active *piggyBac* transposase (Grossman et al., 2000; Lobo et al., 1999).

Five *pMos3xP3EGFP-pBac* transposase-expressing transgenic lines (C09, I03, I12, J02, J26) were created. The patterns of inheritance of the 3xP3EGFP marker gene were consistent with each line containing a single insertion of the vector. Transposable element display confirmed the presence of a single insertion in each line (data not shown). As expected when using the 3xP3 promoter, *Mos3xP3EGFP-pBac* individuals had EGFP marker gene expression in the larval and adult brain and optic stalk (Berghammer et al., 1999). However, the phenotypic expression patterns differed notably among the lines in both the levels and patterns of EGFP expression indicating the position-dependent transgene expression similar to that seen in the case of *cn* gene expression in reporter lines (Coates et al., 1998; Jasinskiene et al., 1998; Lobo et al., 2002; Wilson et al., 2003). The *piggyBac* transposase coding region was amplified from the genomic DNA of each helper line, cloned and sequenced to assess its integrity. The sequence of the transposase transgene in these transgenic lines revealed no mutations, indicating that the *hsp::piggyBac* transposase gene would function as a source of active *piggyBac* transposase 594 amino acids in length. The expression of the *piggyBac* transposase transgene was confirmed in transgenic lines by reverse transcriptase PCR (data not shown).

Four additional lines were created (29, 32, 37A, 38) using *pMos3xP3EGFP-pBac^G*. These lines were similar to lines C09, I03, I12, J02, J26 in that they contained single copies of the

integrated vector but expressed a *piggyBac* transposase gene that contained a single nucleotide deletion in codon 493 that resulted in a premature translational stop codon and a truncated 502 amino acid, nonfunctional transposase.

Somatic stability after direct injection of *piggyBac* transposase-expressing plasmid DNA

Stability of non-autonomous *piggyBac* elements in the soma of *Ae. aegypti* was tested by directly injecting one of two plasmids containing a functional *hsp::piggyBac* transposase transgene. The first plasmid contained only the *piggyBac* open reading frame under the regulatory control of *Drosophila hsp 70* promoter (*pBac/hsΔsst*; Handler et al., 1998) and the second containing the same *piggyBac*-expressing transgene but inserted into the *MosI* vector (*Mos3xP3EGFP-pBac*). A total of 141 G₀ adults (including individuals from each reporter line) developing from injected embryos were analyzed by isolating genomic DNA and performing *piggyBac*-specific transposable element display. Putative somatic transposition events were expected to be seen as transposable element display products with a molecular weight distinct from that arising from the original inserted element and these unique products would have a lower abundance relative to those products arising from the parental element (Guimond et al., 2003; Wilson et al., 2003). No evidence of somatic mobility was seen in these experiments (Figure 2A).

Injecting these same transposase-expressing helper plasmids into a transgenic *D. melanogaster* line containing a single integrated *piggyBac* element resulted in abundant evidence for somatic activity. *D. melanogaster* line 17861 contains a single mini-*white* marked *piggyBac* element on the second chromosome at cytological position 50C. Adults developing from embryos that were injected with helper plasmids had *piggyBac* elements located in numerous new locations as revealed by transposable element display (Figure 2B).

Somatic stability following the creation of 'reporter'/'helper' heterozygotes

To further examine somatic stability, 'reporter'/'helper' heterozygotes were created by mating individuals from the *piggyBac*-containing lines (40D, 40L, MF1.92, MF03 and PE11) with those from the *piggyBac* transposase-expressing helper lines (C09, I03, I12, J02, J26). Adult red-eyed progeny with EGFP expression in the brain ('reporter'/'helper' heterozygotes) were analyzed by transposable element display as described above. 127 heterozygous individuals were analyzed by transposable element display to look for evidence of elements in new locations. While almost all of the heterozygotes examined had no evidence of transposition (Figure 3A), occasionally individuals were observed that contained unique, faint bands relative to the vertically inherited parental element. Such bands indicate the possibility of elements in a new location. Seventeen putative transposition events were detected and further analyzed by being isolated, re-amplified and sequenced. Transposed elements, upon sequencing, will reveal the terminal inverted repeat of *piggyBac* flanked by novel sequences not seen with the original integrated element. Confirmatory analysis showed that none of the 17 unique products resulted from *piggyBac* transposition and these bands arose from the miss-priming, a rare but detectable phenomenon in the protocols used here.

Germ-line stability following the creation of 'reporter'/'helper' heterozygotes

Germ-line stability in the presence of functional *piggyBac* transposase was tested by creating 'reporter/helper' heterozygotes as described above and analyzing their progeny following their mating with non-transgenic individuals from the *kh^w* mutant line (Table 1). Eight 'reporter/helper' combinations were tested and experiments were performed in which the heterozygous parental insects either had, or had not been heat-shocked daily during their larval development at 41 °C for one hour. Of the 20,144 red-eyed adult progeny recovered from these crosses 150 had eye color phenotypes that differed from the parental eye color

(either lighter or darker). These were provisionally considered as transposition events and were further analyzed by transposable element display to test for the presence of *piggyBac* elements in new locations. None of the 150 individuals tested by transposable element display was the product of a germ-line transposition event. The eye color variations seen in these individuals was likely due to intra-strain variation in expression of the *kynurenine hydroxylase* (*cinnabar*) transgene and variation in the nutritional status of the insects (Sethuraman and O'Brochta, 2005).

Germ-line stability in the absence of functional transposase

Germ-line stability was also tested in heterozygotes containing functional 'reporter' elements and non-functional 'helper' elements (29, 32, 37A, 38) that encoded a mutated form of the protein. A total of 11,259 red-eyed progeny were examined and 361 showed eye color variations different from the parental phenotype. One-hundred eye color variants were analyzed by transposable element display and in none had *piggyBac* transposed to a new location.

Methylation status of the terminal *piggyBac* sequences and flanking genomic DNA

Methylation is a major form of epigenetic control of transposable element movement in some organisms (Kunze and Weil, 2002). The role of methylation in controlling insect transposable elements is unknown. The methylation status of the terminal sequences of integrated *piggyBac* elements in *Ae. aegypti* was examined using bisulfite modification mapping, a technique that identifies the location of methylated cytosines (Clark et al., 1994.). Under appropriate conditions bisulfite will convert cytosine to uracil whereas 5-methylcytosine is insensitive. This differential sensitivity can be used to locate methylcytosines in a sequence of genomic DNA (Clark et al., 2006). The terminal 30 bp of the *piggyBac* element in line 40L and 258 bp of genomic DNA immediately flanking the element were assessed and no evidence of methylated cytosines was obtained.

Position-dependent *piggyBac* remobilization rates in *D. melanogaster*

Five *piggyBac*-containing *D. melanogaster* lines from the Bloomington Stock Center (stock numbers 17861, 17975, 18050, 18256, 18759) containing the *mini-white* genetic marker were crossed to a *piggyBac* transposase-expressing 'helper' line (8285). The *piggyBac* elements within these five lines had unknown remobilization characteristics and were distributed on the X chromosome and chromosome 2. Heterozygous adults containing the *w⁺*-containing 'reporter' element and the 'helper' element were scored for the presence or absence of eye-color mosaicism resulting from *piggyBac* transposition and excision in somatic tissue. A 35-fold range in the frequency of somatic mosaicism was observed. The lines showing the fewest number of heterozygous individuals with mosaic eye color were 18050, 18759 and 17975 with 2.7%, 4.9% and 7.8%, respectively (an average of 250 heterozygotes scored in each case). Lines 18256 and 17861, when crossed with 'helper' line 8285, resulted in 45% and 95.4% of the progeny with mosaic eyes, respectively. The degree of eye-color mosaicism observed within each eye was also different among the lines. Lines 18256 and 17861 had a high degree of mosaicism within each eye indicating the presence of multiple somatic genotypes. Mosaic eyes in line 18050 had only one or a few small patches of ommatidia with non-parental eye colors indicating low levels of somatic activity. Transposable element display performed on individuals with evidence of mosaicism in the eyes yielded similar results. Reporter/helper heterozygotes involving line 17861 had abundant evidence of *piggyBac* somatic activity as indicated by the large number of *piggyBac* elements observed in different locations within the genome (Figure 3B). Similarly, reporter/helper heterozygotes involving line 18050, in which *piggyBac* showed little evidence of somatic movement based on eye mosaicism, had no evidence of somatic movement based on transposable element display (Figure 3B).

Discussion

Understanding the post-integration behavior of transposable elements will aid in our understanding of the natural history of transposable elements and their potential to be used as gene vectors. The mobility of Class II transposable elements displays both element-specific and host-specific characteristics. For example, Class II transposable elements integrate into specific sequences or “target sites” that are characteristic of the element and remain invariant regardless of the host genome. *piggyBac* elements always integrate into the target sequence TTAA regardless of the host (Fraser, 2000). The rates of element mobility, on the other hand, tend to be quite variable among hosts and depend in yet unknown factors. Transgenic *Ae. aegypti* arise from about 2-5% of the adults developing from embryos that were injected with *piggyBac* gene vectors, while in *Anopheles albimanus* the rate of transformation is 40-50% using the same vector (Fraser, 2000; Perera et al., 2002). In this report the *piggyBac* element in the foreign host *Ae. aegypti* illustrates how the post-integration behavior of an element can be a host-dependent characteristic. In the experiments reported here the germ-line and somatic mobility of integrated non-autonomous *piggyBac* gene vectors was tested in the presence and absence of *piggyBac* transposase in the yellow fever mosquito, *Ae. aegypti*. Approximately 439 germ-lines and 20,000 progeny were examined as well as the soma of over 250 individuals and the remobilization (transposition) of *piggyBac* was never observed. This high degree of element stability was somewhat unexpected since this element’s remobilization potential in two other insect species has proven to be high. In *D. melanogaster* *piggyBac* has been successfully remobilized using a strategy similar to that described here and many integrated elements were found to undergo frequent germ-line transposition (Horn et al., 2003; Thibault et al., 2004). Similarly, *piggyBac* can readily remobilize in the genome of *Tribolium castaneum* in the presence of *piggyBac* transposase, displaying many of the same characteristics displayed in *D. melanogaster* (Lorenzen et al. 2007). Efficient remobilization of integrated *piggyBac* elements may also occur in mammalian cells (Ding et al., 2005). In *piggyBac* remobilization studies in insects rates varied as a function of integration site (Horn et al., 2003; Lorenzen et al., 2007). We have observed similar variability after choosing five *piggyBac*-containing *D. melanogaster* lines from the Bloomington Stock Center that had *mini-white* marked elements at various locations within the genome. Position-dependent transposition appears to be a general feature of transposable elements and is not limited to insects and Class II elements (Berg and Spradling, 1991; Garza et al., 1991; Lisch et al., 1995; Raina et al., 1993). While these effects are well documented the exact nature of the position effects on element remobilization, in most cases, is not known. Chromatin structure, such as the degree of DNA packing and the extent to which the chromosomes are in ‘open’ or ‘closed’ configuration, may limit access of transposase to critical sequences within the element just as transcription factors have limited access to genes located in heterochromatin and highly packed regions. In addition, the transcriptional activity of genomic regions may influence the degree of access of proteins required for transposition and indirectly influence remobilization frequency. The nature and extent of DNA modifications, such as methylation, may also vary regionally within the genome and influence the transposition activity of transposable elements. The degree of DNA methylation in *D. melanogaster* is low, relative to other organisms, however other insects have vertebrate-like levels of methylation (Field et al., 2004). We found no difference between the methylation states of the terminal sequences of integrated *piggyBac* elements and genomic DNA immediately flanking the integration site, and the homologous DNA in a non-transgenic insect.

What is particularly notable about the data reported here are the degree of element stability and the number of elements displaying this stability within *Ae. aegypti*. In this study, five independently inserted *piggyBac* elements in different locations within the genome displayed a very high level of stability in the presence of transposase. In all cases element

remobilization was never detected in either germ-line or somatic tissue. It is possible then that the apparent differences between the post-integration behaviors of *piggyBac* in *Ae. aegypti* compared to its behaviors in *D. melanogaster* and *T. castaneum* are solely quantitative. If this hypothesis is correct, remobilization in *Ae. aegypti* is infrequent perhaps because of severe position effects and more elements in different locations would need to be examined before evidence of transposition could be found. The genome of *Ae. aegypti* is large (approximately 780 Mb or 5x larger than the genome of *D. melanogaster*), has a large amount of repeat sequences and organized differently from that of *D. melanogaster* and *T. castaneum* in that it has a “short interspersed repeat” pattern whereas the latter two species have “long interspersed repeat” patterns. It is possible that the size and organization of the *Ae. aegypti* genome contributes to more severe position effects on element.

A number of pieces of evidence indicate that transposase expression was not a problem in experiments reported here. First, the sequence of the *hsp70::transposase* transgene contained within the integrated *Mos1* gene vectors revealed no mutations in the helper lines. Second, the *hsp70::transposase* transgene contained within the *Mos1* vector was transcriptionally active as revealed by reverse transcriptase PCR performed on RNA isolated from ‘helper’ lines (data not shown). We are also confident that the *hsp70* promoter from *D. melanogaster* is active in the germ-line of *Ae. aegypti* because most so-called “helper” plasmids used during the creation of transgenic *Ae. aegypti* in the past have relied on this promoter and the successful creation of stably transformed lines is dependent upon the expression of a transposase-expressing helper gene in the germ-line (Coates et al., 1998; Jasinskiene et al., 1998; Kokoza et al., 2000; Kokoza et al., 2001; Lobo et al., 2002; Pinkerton et al., 2000; Wilson et al., 2003). We also demonstrated that the *hsp70::transposase* helper gene was capable of producing functional transcript by demonstrating our ability to drive somatic transpositions of *piggyBac* after transiently expressing transposase from *pMos3xP3EGFP-pBac* vectors injected into *piggyBac*-containing *D. melanogaster*. *piggyBac* transpositions were also observed when *pMos3xP3EGFP-pBac* was used as a helper plasmid in a plasmid-based *piggyBac* transposition assays in *D. melanogaster* embryos. Finally, this same *hsp70::transposase* transgene was also used by Lorenzen et al (2007) to supply functional *piggyBac* transposase to integrated *piggyBac* elements in *T. castaneum*. Taken together we suggest that these data make it very unlikely that transposase expression was limiting *piggyBac* mobility in *Ae. aegypti*.

While it is possible that *piggyBac* remobilization in *Ae. aegypti* is merely quantitatively different from *D. melanogaster* and *T. castaneum*, it is also possible that there are more substantial qualitative differences in the suitability of *Ae. aegypti* as a host for introduced transposable elements. There are some data that suggest that the transposition reactions of some introduced Class II transposable elements in *Ae. aegypti* is different from that observed in *D. melanogaster* and the few other insect species that have been repeatedly transformed. The four common transposable element vectors (*Minos*, *Mos1*, *piggyBac* and *Hermes*) have displayed canonical cut-and-paste transposition activity in all of the species in which they have been used except for *Ae. aegypti*. The *Hermes* element has exhibited the most extreme examples of altered integration behavior in *Ae. aegypti*. While this element transforms *Ae. aegypti* as readily as other vectors, all of the integrated *Hermes* vectors in this species that have been analyzed appear to have undergone a non-canonical transposition reaction in which large amounts of plasmid DNA flanking the vector are integrated along with the *Hermes* element (Jasinskiene et al., 2000). Despite the non-canonical nature of these integrations they were transposase-dependent. Integrated *Hermes* elements in *Ae. aegypti* also display a high degree of stability with germ-line transpositions never being detected in the presence of transposase (O’Brochta et al., 2003). Somatic remobilizations of *Hermes*, however, are observed and these new transposition events occur via canonical cut-and-paste transposition reactions (O’Brochta et al., 2003). Similarly, some of the primary

integration events involving *Mos 1* and *piggyBac* elements have also involved non-canonical recombination reactions resulting in the integration of plasmid DNA sequences in addition to vector DNA (Adelman et al., 2004; Coates et al., 2000). Two *piggyBac* reporter lines were created during this study but were not used in remobilization studies because of the non-canonical nature of the integration events (unpublished data but see figure 2). Such non-canonical events have not been described in *D. melanogaster* and other insect species in which these vectors have been used. By these criteria *Ae. aegypti* appears to be a qualitatively different host compared to other insects that have been transformed. It is interesting to speculate if the disturbances in the transposition reactions associated with element integration and the generally poor potential for elements to remobilize in *Ae. aegypti* are related to an endogenous system for resisting and combating invasion by foreign genomes such as viruses and transposable elements. Recently the important role of RNAi in response to virus infection in *Ae. aegypti* has been described illustrating that this genome protection system is fully developed in this insect (Sanchez-Vargas et al., 2004). While it is intriguing to consider the possibility that *Ae. aegypti* may have an active anti-transposon surveillance system that interferes with post-integration remobilization, preliminary experiments in which the *Ae. aegypti Dicer2* gene was knocked out by direct injection of double-stranded homologous RNA did not result in detectable somatic movement of *piggyBac* in the presence of transposase (N. Sethuraman and D. O'Brochta, unpublished data). Additional studies of this type are needed, targeting other genes involved in silencing, to fully explore the role of RNAi in regulating the movement of integrated gene vectors.

The absence of element mobility following the integration of gene vectors has important practical implications. First, functional genomic studies and certain proposed insect control strategies rely on the construction of stable genotypes. The high level of element stability reported with *piggyBac* enhances the utility of this gene vector in *Ae. aegypti*. Recently systems for stabilizing integrated gene vectors in insect genomes have been described that rely on secondary recombination events following vector integration (Handler et al., 2004). The high levels of *piggyBac* stability reported here, even in the presence of *piggyBac* transposase, reduce the need for these secondary modification procedures to achieve vector stability in this species.

On the other hand, there is an interest in developing transposon-based gene spreading technologies that would be used to introduce transgenes that interfere with pathogen and parasite development in natural populations of disease transmitting insects. For such applications transposable elements are needed with high rates of remobilization with a strong tendency to increase in copy number. It would appear that *piggyBac*, as configured and used in the experiments presented here, will not be useful as a genetic drive agent in *Ae. aegypti*.

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Figure 1. *pMos3xP3EGFP-pBac* is a source of functional *piggyBac* transposase

A plasmid-based transposition assay was performed in *D. melanogaster* embryos. Target plasmids with integrated *piggyBac* elements were selected and the integration site determined by DNA sequencing. The target plasmid, pGDV1, is represented linearly with all TAA target sites indicated with diamonds and the position of the chloramphenicol resistance gene used during the selection of transposition products is shown (Cam^R). The position (x-axis), number (y-axis) and relative orientation of *piggyBac* transposition events are shown (circles).

Figure 2. Somatic remobilization of *piggyBac* by helper plasmid injection

A. Individual embryos of *piggyBac*-containing *Ae. aegypti* line 40D were injected with helper plasmid *pMos3xP3EGFP-pBac*. Individual adults developing from injected embryos were processed for *piggyBac* transposable element display. The *piggyBac* element present in 40D individuals is displayed as a 240 bp band. The *piggyBac* sequences contained on the helper plasmid also have the transposable element display priming sites and a PCR product approximately 300 bp reveals the presence of helper plasmid in injected embryos. The sizes of the molecular weight standards (mw) are indicated in base pairs. Note that some individuals contain a second *piggyBac* element. This element (*) is known to be dysfunctional and was not the object of this study. **B.** Individual *D. melanogaster* embryos of the *piggyBac*-containing line 17861, containing a single element, were injected with the helper plasmid *pMos3xP3EGFP-pBac*. The resulting adults were processed for *piggyBac*

transposable element display. Non-injected 17861 are shown as controls (C) and show faint non-specific PCR products. The *piggyBac* element in the genome of 17861 results in a transposable element display-product approximately 1600 b in length and is poorly displayed under the conditions used here. The *piggyBac* sequences contained on the helper plasmid have the transposable element display priming sites and a PCR product approximately 300 bp reveals the presence of helper plasmid in injected embryos. The numerous bands in injected insects are *piggyBac* elements in new locations resulting from transposition as shown following band isolation, re-amplification and DNA sequencing. The sizes of the molecular weight standards (mw) are indicated in base pairs.

A

B

Figure 3. Somatic remobilization of *piggyBac* in individuals heterozygous for ‘reporter’ and ‘helper’ transgenes

A. The genotypes are indicated. 40L, 40D, PE11, MF1.92 and MF03 refer to *piggyBac*-containing ‘reporter’ elements. All transposase-expressing ‘helper’ lines (I03, J02, I12 and C09) result in the same 300bp PCR product being produced during the transposable element display procedure regardless of the genomic position of the integrated *Mos3xP3EGFP-pBac* element. *piggyBac* elements at locations other than those of elements inherited from the parental insects were not observed. The sizes of the molecular weight standards (mw) are indicated in base pairs. **B.** Heterozygous *D. melanogaster* with mosaic eyes and containing a single copy of a *piggyBac* ‘reporter’ element (17861, 18050 and 18256) and a ‘helper’

element (8285) were analyzed by transposable element display. Evidence of somatic activity is apparent in heterozygotes of 17861 and 18256 but not 18050. The *piggyBac* element in the genome of 17861 results in a transposable element display product greater than 1kb and is not visible on these displays (*).

Table 1
‘Reporter’/‘Helper’ heterozygotes

	<i>Crosses</i>	<i>Red-eye progeny</i>	<i>Eye-color variants</i>
1.	40D × C09	9516	53
2.	40D × I03	5974	54
3.	40D × I12	169	7
4.	40D × J02	184	-
5.	40L × C09	1583	12
6.	40L × I03	2254	24
7.	40L × I12	110	-
8.	40L × J02	354	-
	Total	20144	150