Published in final edited form as:

Insect Biochem Mol Biol. 2013 October ; 43(10): 899–906. doi:10.1016/j.ibmb.2013.06.009.

IPB7* transposase behavior in *Drosophila melanogaster* and *Aedes aegypti

Jennifer A. Wright¹, Ryan C. Smith², Kefong Xie³, Nancy L. Craig³, and Peter W. Atkinson^{1,2,4}

¹Department of Entomology, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 212205-2185, USA

²Cell Molecular and Developmental Biology Graduate Program, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 212205-2185, USA

³Department of Molecular Biology and Genetics, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 212205-2185, USA

⁴Center for Disease Vector Research, Institute for Integrative Genome Biology, University California Riverside, Riverside, CA 92521, USA

Abstract

Transposons are used in insect science as genetic tools that enable the transformation of insects and the identification and isolation of genes through their ability to insert in or near to them. Four transposons, *piggyBac*, *Mos1*, *Hermes* and *Minos* are commonly used in insects beyond *Drosophila melanogaster* with *piggyBac*, due to its wide host range and frequency of transposition, being the most commonly chosen. The utility of these transposons as genetic tools is directly proportional to their activity since higher transposition rates would be expected to lead to higher transformation frequencies and higher frequencies of insertion throughout the genome. As a consequence there is an ongoing need for hyperactive transposases for use in insect genetics, however these have proven difficult to obtain. *IPB7* is a hyperactive mutant of the *piggyBac* transposase that was identified by a genetic screen performed in yeast, a mammalian codon optimized version of which was then found to be highly active in rodent embryonic stem cells with no apparent deleterious effects. Here we report the activity of *IPB7* in *D. melanogaster* and the mosquito, *Aedes aegypti*. Somatic transposition assays revealed an increase in *IPB7*'s transposition rate from wild-type *piggyBac* transposase in *D. melanogaster* but not *Ae. aegypti*. However the use of *IPB7* in *D. melanogaster* genetic transformations produced a high rate of sterility and a low transformation rate compared to wild-type transposase. This high rate of sterility was accompanied by significant gonadal atrophy that was also observed in the absence of the *piggyBac* vector transposon. We conclude that *IPB7* has increased activity in the *D. melanogaster* germ-line but that a component of the sterility associated with its activity is independent of the presence of the *piggyBac* transposon.

© 2013 Elsevier Ltd. All rights reserved.

Corresponding author: Peter W. Atkinson Department of Entomology and Center for Disease Vector Research University of California 900 University Avenue Riverside, CA 92521 USA Phone: 951 927 4782 peter.atkinson@ucr.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

piggyBac; transposable element; hyperactive; *Drosophila*; *Aedes*

1.0 Introduction

DNA transposons are small pieces of DNA that can move within and between genomes. Their mobility properties have led to some of them being used as genetic tools in a number of model organisms in which they can integrate themselves as well as transgenic cargo. Improving the performance parameters of transposons is critical to their further development as more efficient genetic tools such as gene and enhancer trapping, and the stable introduction of RNAi constructs. The *piggyBac* transposon from the cabbage looper, *Trichoplusia ni*, has proven to be a highly efficient transposon in invertebrates, vertebrates and protozoa and is currently used in gene therapy and transposon-based strategies to identify mammalian genes (Ding et al., 2005; Wilson et al., 2007). *piggyBac* integrates into TTAA nucleotide sites and due to its unique staggered cleavage upon excision, leads to the perfect repair of donor sites meaning that it can be introduced into a genome to achieve a desired goal and then precisely removed (Elick et al., 1996; Mitra et al., 2008; Wang and Fraser, 1993). Another advantage of *piggyBac* is its ability to insert large (14.3 – 100 kb) segments of genomic material without a loss of efficiency (Ding et al., 2005; Li et al., 2011). *piggyBac* has proven its capacity as a gene vector in a wide variety of insects including, but not limited to: *Ceratitis capitata*, *Aedes aegypti*, *Anopheles stephensi*, *Anopheles albimanus*, *Anopheles gambiae* and *Tribolium castaneum* (Grossman et al., 2001; Handler et al., 1998; Kokoza et al., 2001; Lorenzen et al., 2003; Nolan et al., 2002; Perera et al., 2002). While transformation of mosquitoes has succeeded, frequencies remain low for all but *An. albimanus*, typically less than 4% for *Mos1*, 7% for *piggyBac* and 6% for *Hermes* in *Ae. aegypti*, the mosquito species most amenable to genetic manipulation (Adelman et al., 2002). For *An. albimanus* the single report of genetic transformation described four independent lines generated using the *piggyBac* transposon with transformation frequencies ranging from 20 to 43% (Perera et al., 2002). The reason for this high rate of transformation for *An. albimanus* alone remains unknown but the range of transformation frequencies across mosquito species illustrates that a number of factors, such as experience of the researcher, the cargo capacity of the transposon, the genetic marker used, and the ease with which fertilized eggs can be obtained and manipulated can affect the efficiency of the procedure. One strategy that can supplant this variability is to generate transposases that have hyperactivity.

piggyBac preferentially inserts within genes, with 50% to 67% of insertions occurring within transcriptional units in human and mouse systems and is not sensitive to over-production inhibition in which elevated levels of the transposase causes decreased transposition (Ding et al., 2005; Grabundzija et al., 2010; Li et al., 2012; Wilson et al., 2007). A bias towards insertion into introns within transcriptional units in *D. melanogaster* has also been observed (Hacker et al., 2003).

There is a precedent for increasing transposition rates in DNA transposons through mutagenesis. The *Tc1/mariner* transposon *Sleeping Beauty* (*SB*) was initially synthesized from the alignment of inactive transposons belonging to the *salmonid* subfamily of elements (Ivics et al., 1997). Endogenous DNA transposons from this family are completely inactive in vertebrate genomes and are thought to have lost functional ability many millions of years ago, with only their inactive remnants found throughout vertebrate genomes. This restoration of activity demonstrated that disabled transposons can be restored to activity through the manipulation of their transposon sequence with hyperactive forms of *SB* being

subsequently isolated through conventional mutagenesis and molecular evolution strategies (Baus et al., 2005; Mates et al., 2009). A corollary is that currently active transposable elements can be mutated to achieve higher transposition frequencies. Progress in generating hyperactive transposons for use in insect transgenesis has been, at best, slow. A hyperactive form of the *P* transposase was obtained by selectively altering serines to alanines testing the hypothesis that inhibition of any phosphorylation may increase activity (Beall et al., 2002). One mutant, S129A, was obtained which had a 172% increase in excision frequency in embryos and a 3- to 5- times increase in transformation frequency (Beall et al., 2002). Several hyperactive forms of the *Mos 1* and *Himar* transposase were obtained using a papillation assay in *E. coli*, however none retained any activity when tested in *D. melanogaster* (Lampe et al., 1999).

Recently a mutated *piggyBac* element named *IPB7* was obtained through using error-prone PCR mutagenesis and a high throughput genetic screen for hyperactivity performed in yeast (Yusa et al., 2011). Based on the analysis of 18 mutants in yeast, five mutants (containing a total of seven mutations) were introduced using site-directed mutagenesis into the mPBBase ORF which had previously been codon optimized for mammalian expression to generate hyPBBase (Cadinanos and Bradley, 2007; Yusa et al., 2011). As seen in yeast, hyPBBase retained hyperactivity in mouse embryonic stem cells (ESCs) displaying a 17-fold increase in excision and 9-fold increase in integration without affecting the genomic integrity of these cells (Yusa et al., 2011). HyPBBase was subsequently shown to increase the efficiency of gene transfer into human HeLa, HEK293 and T cells (Doherty et al., 2012). *IPB7* and hyPBBase contain the same seven amino acid substitutions: (I30V, G165S), S103P, M282V, (S509G, N570S) and N538K. However *IPB7* retains the codon bias of the original *piggyBac* transposon from *T. ni*. Here we examined whether *IPB7* is capable of achieving increased levels of transposition in somatic and germ-line cells of *D. melanogaster* and *Ae. aegypti*.

2.0 Materials and Methods

2.1 Lab strains

D. melanogaster strain C-S;*w* was used in all fly experiments; the Orlando strain of *Ae. aegypti* was used in all mosquito experiments. This strain is maintained at the University of California, Riverside under standard insectary conditions as described (Munstermann L. E., 1997) with the one exception that larvae are fed on a diet of Milkbone Original Dog Biscuits mixed with Red Star Specialty Nutrex 55. Adults were fed on a 10% sucrose solution and females blood fed on mice. The mosquitoes are maintained at 26° C with a photoperiod of 16:8 (L:D).

2.2. Plasmid Constructions

2.2.1 *piggyBac* donor plasmid—pBacGoEGFP was constructed by amplifying the left and right ends of *piggyBac* from the [3xp3-EGFPafm] *piggyBac* transposon (Horn and Wimmer, 2000). The left end, its flanking target site duplication (TSD), a portion of flanking DNA and part of the *piggyBac* ORF was amplified with the primers SacII-pBac LE F (5'-ATACCGCGGTCTTTTAAACCCTAGAAAGATAGTCTGCC-3') and XbaI-pBac LE R +ORF (ATATCTAGAGCTCATCGTCTAAAGAACTACCC- 3'). Next the right end, TSD and flanking DNA were amplified with the primers KpnI-pBac RE R (5'-ATAGGTACCCTATTATTAACCCTAGAAAGATAATCATATTGTG-3') and the left and right portions were digested using restriction sites incorporated into the PCR products and inserted into pBluescript II KS+ using the *SacII-XbaI* and *KpnI-XhoI* sites. The resulting plasmid pBSpBacLR was then linearized through digestion with *XbaI* as a means of incorporating a *NheI* linearized pGENToriEGFP plasmid thus creating pBacGoEGFP. pGENToriEGFP was created through the excision of the lacZα ORF from the pGENToria

plasmid by digestion with *Bam*HI and *Nhe*I and replaced by a *Bam*HI/*Nhe*I fragment containing the EGFP ORF. The *Bam*HI/*Nhe*I EGFP fragment was generated through PCR amplification of the pBac[3xp3-EGFPafm] ORF using the primers *Bam*HI EGFP start (5'-ATAGGATCCACCGGTCGCCACCATGGTG-3') and *Nhe*I EGFP stop (5'-ATAGCTAGCTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGG-3'). Following PCR, the product was digested with *Bam*HI and *Nhe*I in preparation for ligation.

2.2.2 piggyBac helper plasmid—The *piggyBac* helper construct was made by the removal of the transposase ORF from pGalsHYPBASE (Yusa et al., 2011) using *Spe*I-*Xho*I and ligation into the vector pKH70new digested with the same enzymes. pKH70new is an altered form of plasmid pKhsp70 (Arensburger et al., 2005). The hsp70 promoter was PCR amplified from pKhsp70 using the primers *Eco*RI Hsp Prom F: (5'-GATGAATTCGTGCTCTCGTTGGTTCGAGAGAG-3'), *Kpn*I Hsp Prom R: (5'-GATGGTACCACTAGTCTGCAGATTGTTTAGCTTGTTCAGCTG-3'). The hsp70 3' UTR was amplified using the primers *Pst*I Hsp Term F: (5'-AATCTGCAGCTCGAGTCGACTAAAGCCAAATAGAAAATTATTCAGTTC-3') and *Hind*III Hsp Term R: (5'-GATGAAGCTTGGATCTAAACGAGTTTTTAAGCAAACCTCACTCC-3'). pK19 vector and PCR products were digested with *Kpn*I and *Eco*RI, and ligated resulting in pK19hsp, which contained the hsp70 promoter. Next, the hsp70 3' UTR product and pK19hsp were digested with *Pst*I and *Hind* III and ligated resulting in pKH70new. pGalHYPBASE was derived from the p414GalS plasmid with the addition HYPBASE transposase between base pair 3245 and 3246.

2.2.3 piggyBac vector plasmid used for transformations—The pBac[3xP3-EGFP]af plasmid was previously described (Horn and Wimmer, 2000).

2.2.4 Hermes plasmids used for transposition assays—pHDG1 was the *Hermes* donor plasmid used in transposition assays. pHDG1 includes the complete left and right ends of the *Hermes* transposon with none of the *Hermes* open frame included. The left end was amplified from pHERKS3, also called pHermesKSacOα (Sarkar et al., 1997), by PCR using the primers HL8bpSac (5'-TGAGAGCYCGYCGTATCAGAGAACAACAACAAGTGGCTTATTTG-3') and HLinternalRxbA (5'-TGATCTAGACTCTACTACTGCTTAAACTCTTTGAGGATGAACAG-3'). The product was then digested with *Sac*I and *Xba*I and cloned into pBLuescript SK+ producing pSHLfull. The right end was amplified from pHERKS3 by PCR with the primers HRinternalFxbA (5'-TGACTCGAGGACTGGAAAAATTTAAGTTTAAAGAAGC-3') AND HR8bpRKpn (5'-TGAGGTACCATACAGACAGAGAACTTCSAACAAGTCACAGGC-3') and digested with *Xho*I and *Kpn*I and cloned into pBLuescript SK+ digest with the same enzymes to produce pBSHRfull. pBSHRfull was digested with *Kpn*I and *Xho*I to excise the right end which was cloned into pBSHLfull which was digested with the same enzymes to produce pBSHLRfull. pBSHLRfull was digested with *Xba*I and ligated with pGentOriα digested by *Nhe*I to produce the donor plasmid pHDG1

pGentOriα was made by digesting pK19 with *Nae*I and *Nhe*I to isolate the *E. Coli* replication origin and *LaZa* gene and *Nhe*I site was filled by Klenow fragment. pFastBac ORF (Invitrogen) was digested using *Msc*I and *Sna*BI to isolate the GentR ORF. The two fragments were then ligated together to create pGentOriα. pKSHH14 was the *Hermes* helper plasmid used and has previously been described (Sarkar et al., 1997). pGDV1 has been described previously (Sarkar et al., 1997).

2.2.5 Transposition assays in *D. melanogaster* and *Ae. aegypti*—*IPB7*

transposase was tested by co-injecting the appropriate helper and donor plasmids along with the *Hermes* donor, *Hermes* helper and pGDV1 as a target. This injection mix contained the following: 250ng/ul of *pBacGoEGFP* donor, 250ng/ul of the mutant *IPB7* helper-transposase, 250ng/ul of *Hermes* donor, 250ng/ul of *Hermes* helper-transposase and 1000ng/ul of pGDV1 as a donor plasmid. *D. melanogaster* embryos were heat shocked at 37°C for 1 hr the day following microinjection; *Ae. aegypti* embryos at 42°C for the same time period. By using *Hermes* in the assay along with *IPB7* we could compare the number of transposition events of both *piggyBac* transposases relative to the number of *Hermes* transposition events thereby allowing normalization between injections and experiments. Protocols for microinjection of embryos and plasmid rescue have been previously described (Sarkar et al., 1997).

2.2.6 Insect Transformation—Transformation procedures for *D. melanogaster* and *Ae. aegypti* were described previously (Jasinskiene et al., 1998; Rubin and Spradling, 1982). *D. melanogaster* embryos were injected with helper plasmid concentrations of 250, 50 or 25 ng/ul and heat-shocked the following day as described above. *Ae. aegypti* adults were blood fed on anaesthetized mice for 10 minutes. 48 hrs later, females were provided with a small tub of water with Whatman paper filter immersed in it and allowed to lay eggs for 30 minutes. The embryos were then aligned on double stick tape and mounted on a plastic slide and covered with halocarbon oil 27. After microinjection the oil was washed away and the slides were placed in a pan of deionized H₂O. 16-18 hours post injection the embryos were heat shocked at 42° C for one hour.

2.2.7 Ovary dissections—*D. melanogaster* ovaries were dissected two weeks after GO adults were crossed. Mosquito ovaries were dissected out after the female had imbibed three blood meals. Each *Aedes* female had the opportunity to lay eggs after the first two blood feedings and if she did not produce viable offspring she was considered sterile. Dissected ovaries were placed in a glycerol based mounting fluid and photographed using a Zeiss Axioskop2.

3.0 Results

3.1 *IPB7* shows no significant increase in somatic transposition in *D. melanogaster*

We evaluated the somatic transposition rate of *IPB7* in *D. melanogaster* using a normalized interplasmid transposition assay in which the activity of the *IPB7* transposase was compared with an internal control transposase, each transposase being placed under the control of the *D. melanogaster* hsp70 promoter.

IPB7 generated a transposition frequency of 3.14×10^{-3} while the transposition frequency of the wild-type *piggyBac* transposase was 1.14×10^{-3} which was consistent with the frequency of wild-type *piggyBac* transposition in this insect (Table 1) (Lobo et al., 1999). While this apparent difference in activities between *IPB7* and wild-type *piggyBac* transposase was negligible, it was large when normalized to the frequency of *Hermes* transposition measured in the same experiments with an approximate 10-fold difference in activity (Table 1 and Fig. 1). Since *PBase* also showed clear hyperactivity in both yeast and mammalian cells we were interested to determine whether the same seven mutations present in *IPB7* may lead to increased germ-line activity in *D. melanogaster*, which is not measured in the somatic interplasmid transposition assays.

3.2 *IPB7* produces a high rate of sterility in *D. melanogaster*

Germ-line transformation was achieved by injecting the *IPB7* helper plasmid and the vector plasmid pBac[3xP3-EGFP]af into *D. melanogaster* embryos. Wild-type *piggyBac* transposase and the same *piggyBac* vector were injected as controls at the same plasmid concentrations.

A total of 209 embryos injected with wild-type *piggyBac* transposase survived to adulthood (a survival rate of approximately 43%) and of those 192 (92% of G0 crosses) generated progeny upon backcrossing (Table 2). Screening the G1 progeny for GFP expression revealed that approximately 10% of the wild-type *piggyBac* transposase crosses produced transgenic offspring. Injections with *IPB7* resulted in 239 embryos surviving to adulthood (a survival rate of 42%) of which 145 were fertile (67% of G0 crosses). Surprisingly, only 0.68% of the *IPB7* fertile crosses resulted in transgenic progeny (Table 2). These data are in contrast to the activity of *IPB7* in somatic cells however the decline in transformation rate is matched with an increase in sterility. The overall rate of sterility found with wild-type *piggyBac* transposase was approximately 9% while it increased to approximately 39% with *IPB7* (Table 2). This significant increase in sterility associated with the use the *IPB7* could be due to the transposase causing higher or sustained rates of insertion of *piggyBac* thereby increasing its mutagenic effect, a direct effect of the transposase on chromosomal DNA such as double strand breaks that are repaired inefficiently, or a combination of both. To distinguish between these possibilities, we injected the *IPB7* helper plasmid alone into embryos and measured G0 sterility. These experiments yielded a sterility rate of 23.0% indicating that increased integration of the transposon alone is probably not the only explanation of the increased sterility (Table 2).

The concentration of helper plasmids used in these experiments was 250 ng/ul. We next performed two series of transformation experiments with *IPB7* helper plasmid concentrations at 5-fold and 10-fold less in order to determine if sterility could be reduced with a concomitant increase in transformation frequency. There was a 4% decrease in sterility when the helper plasmid concentration was lowered 5-fold to 50 ng/ul and a further 15% decrease when lowered 10-fold to 25 ng/ul indicating that sterility levels were inversely correlated with the amount of *IPB7* transposase plasmid injected into embryos. However no transgenic progeny were obtained from any of the six independent experiments performed at these lower helper plasmid concentrations indicating that, at least at these concentrations, sterility and the production of transgenics appeared to be unlinked.

The high sterility levels observed led us to investigate the physiological effects of *IPB7* in G0 females. Gonadal dystrophy has been intimately associated with hybrid dysgenesis that arises through the release of suppression of *P* element mobility when *P* element containing sperm fertilize an egg devoid of *P* elements in its genome (Bingham et al., 1982). More recently piRNAs have been proposed to be the actual basis of hybrid dysgenesis with the deficiency of these in the egg allowing *P* elements present in the sperm genome to move in the fertilized zygote resulting in insertional mutagenesis, gonadal atrophy and hybrid dysgenesis (Brennecke et al., 2008; Jensen et al., 2008). To determine if the sterility observed in G0 crosses arising from using *IPB7* was accompanied by gonadal atrophy we dissected ovaries from a subset of sterile female *D. melanogaster* that were either injected with wild-type *piggyBac* transposase or *IPB7* from the transformation experiments using 250 ng/ul of transposase (Fig. 2). 7/9 ovaries taken from sterile flies injected with wild-type *piggyBac* were smaller in size yet still maintained the overall structure of the fertile ovary (Fig. 2C). Two displayed signs of atrophy. However 30/32 ovaries dissected from sterile flies injected with *IPB7* were completely atrophied with only the peritoneal sheath that surrounds the ovaries remaining (Fig. 2D). The remaining two had smaller ovaries. 18/18

ovaries from the sterile females injected with *IPB7* at a helper plasmid concentration of 250 ng/ul alone also displayed the same type of atrophy (Fig. 2E).

3.3 The activity of *IPB7* in *Ae. aegypti*

Interplasmid transposition assays using *IPB7* revealed no significant difference in its activity in *Ae. aegypti* relative to that obtained using wild-type transposase (Table 3 and Fig. 1). We attempted germ-line transformations using the same helper and reported plasmids as used for *D. melanogaster* but did not recover any transgenic progeny from 21 fertile crosses (Table 4). As was seen in *D. melanogaster* we did observe a high degree of sterility (77%) and so examined the ovaries of 11 G0 females and compared them to ovaries dissected from adult female mosquitoes of the same age. All ovaries from these G0 females displayed the same type of atrophy seen in corresponding *D. melanogaster* females (Fig. 3). While we did not perform injections with helper plasmid alone, we concluded that *IPB7* had the same type of behavior in *Ae. aegypti* as it did in *D. melanogaster*.

4.0 Discussion

The use of transposons as genetic tools has led to interest in generating hyperactive forms of them to further increase their efficiency. These gain of function mutations are rare and their usefulness can be balanced against their hyperactivity being so high that their mutagenic properties exceeds their ability to be used effectively. In insect biotechnology, gains have been modest. A hyperactive form of the *P* transposase obtained through directed mutagenesis of serine residues potentially targeted by kinases showed a small increase in transposition in somatic assays with no substantial increase in germ-line transformation frequency (Beall et al., 2002). *Mos1* and *Himar* hyperactive mutants selected in *E. coli* using papillation assays had no activity at all when tested in *D. melanogaster* (Lampe et al., 1999). In contrast hyperactive mutants of the *Sleeping Beauty* transposase from teleost fish generated through site directed mutagenesis and genetic screens have shown repeated high rates of transposition in human cell culture illustrating that such mutants can be obtained and used to improve existing transposon-based genetic techniques (Mates et al., 2009). No activity of *Sleeping Beauty* has, for reasons unknown, been demonstrated in *D. melanogaster*.

The properties of the *piggyBac* transposon make it an obvious target for hyperactive mutagenesis strategies and, given *piggyBac*'s utility as a gene vector across the animal kingdom, it is reasonable to propose that a hyperactive form of its transposase that functions in vertebrates may well preserve this activity in invertebrates, especially since this transposon was originally identified in an insect. *IPB7* was isolated through screening for hyperactive mutants in a yeast-based transposition assay followed by site-directed mutagenesis into the original *piggyBac* single open reading frame that contained seven amino acid substitutions relative to the wild-type transposase (Yusa et al., 2011). HyPBBase, a form of *IPB7* codon optimized for mammalian expression, showed a 17- and 9-fold increase in excision and transposition frequency respectively in mouse ESCs and in human cells without any obvious detrimental effects directly attributable to the transposase (Doherty et al., 2012; Yusa et al., 2011).

To determine if hyperactivity was retained in insects, we evaluated the activity of *IPB7* in the model insect *D. melanogaster* and in *Ae. aegypti*, a vector of several human pathogens. We concluded that *IPB7* had higher levels of activity than wild-type *piggyBac* transposase in interplasmid-based assays performed in somatic cells of *D. melanogaster* but not *Ae. aegypti*. However, *IPB7*'s expression in germ-line cells led to high rates of sterility, accompanied by gonadal atrophy, in both species. In *D. melanogaster* this was also

associated with a low rate of genetic transformation while our results in *Ae. aegypti* suggest that *IPB7* would not be likely to produce a high frequency of genetic transformation.

Indeed, the germ-line transformation experiments in *D. melanogaster* revealed that the *IPB7* transformation rate was dramatically decreased compared to wild-type *piggyBac* transposase. The overall wild-type transformation rate using wild-type *piggyBac* transposase was approximately 10% compared to *IPB7* which had an overall transformation rate of under 1%. Wild-type *piggyBac* transposase generated a sterility frequency of approximately 11% while *D. melanogaster* embryos injected with both the *IPB7* helper and the vector plasmids led to a sterility rate of approximately 40%. Reducing the concentration of *IPB7* by 5- and 10-fold did lead to a reduction in the frequency of sterility but did not generate any transgenic progeny in the following generation in multiple experiments.

Injecting the *IPB7* transposase at 250 ng/ul without a donor element generated a sterility rate of 23% suggesting that the overall sterility rate of approximately 40% seen in the presence of the *piggyBac* element was due both to the increased mutagenic action of the transposon as well as a direct effect of the transposase on the genomic DNA. The latter could be due to the mobilization of any *piggyBac* end-like sequences incumbent in the genome or due to some direct enzymatic effect of *IPB7* on chromosomal DNA. The *D. melanogaster* genome does not contain any *piggyBac* transposons however a *piggyBac*-like sequence called *looper1* has been identified and has three partial copies in the euchromatic DNA (Kaminker et al., 2002). Analysis of these *looper1* sequences did not reveal any to contain terminal sequences that might be recognized by *IPB7*. Furthermore analysis of a piRNA library generated from *D. melanogaster* ovaries showed several piRNAs targeting these *looper1* sequences suggesting that *looper1* may be inactivated by this pathway, which is proposed to silence transposon activity (Brennecke et al., 2007; Gunawardane et al., 2007). These observations suggest that mobilization of *looper1* sequences by *IPB7* is not responsible for the sterility arising from the presence of this transposase alone.

Taken together, the failure to generate transgenic progeny when *IPB7* transposase concentrations were lowered combined with the ability of this transposase to cause sterility in the absence of supplied *piggyBac* transposon suggest that the transposase may indeed be hyperactive but may also have acquired a dominant gain-of-function activity that results in sterility.

Increased sterility in both species was associated with complete gonadal atrophy in G0 females. Gonadal atrophy is a hallmark of hybrid dysgenesis which has been best characterized for the *P* element of *D. melanogaster*. As discussed above, the *piggyBac* element has not been reported in *D. melanogaster* C-S, *w*⁺ and only four *piggyBac* family elements with a total of 31 copies have been found in the reference genome (Liverpool) strain of *Ae. aegypti* although it is formally possible that the Orlando strain used in our injections has a different complement of these transposons.

The *hsp70* promoter used to control *IPB7* is expressed in both the germ-line and somatic tissues. In order to determine any other mutagenic affects of *IPB7*, sterile individuals were examined to count total appendages, abdominal, segments, mouthparts and wing structure. Testes were also dissected. However, the relatively small size of this organ made distinguishing between naturally occurring variation and true mutagenic affects difficult to determine. We could find no other phenotypic manifestations of *IPB7* activity in these individuals. We conclude that either the effect of *IPB7* is confined to the gonadal tissue despite the fact that expression of the gene should be throughout the insect, or there are affects in the somatic tissue that we were unable to detect. Consistent with the former

explanation is our failure to detect an increase in transposition of *piggyBac* in the presence of *IPB7* in somatic cells.

While *IPB7* in its current form is not useful as a genetic tool for the mobilization of *piggyBac*, its generation from the yeast-based screen illustrates that this approach does result in transposases with increased levels of activity and so should be applicable to other transposon systems. The effects we have observed in both *D. melanogaster* and *Ae. aegypti* are significantly more detrimental to the host organism than what was seen in rodent embryonic stem cell culture (Yusa et al., 2011). One explanation for this difference is that cell culture is not an appropriate platform for determining all the possible effects on phenotype that a hyperactive transposase may have on a multicellular organism through development, in which case studying the effects on *IPB7* on living rodents may reveal any pleiotropic effects. Alternatively, but not necessarily exclusively, *IPB7* protein may be more efficiently produced in insects given that *piggyBac* was first detected in an insect.

We do not know the biochemical basis of *IPB7* hyperactivity, nor do we know which of the seven point mutations in it contribute most to this phenotype. The hyperactivity could be due to increased catalytic activity of the enzyme, increased stability, or a combination of both. A component of this does require the presence of the *piggyBac* transposon suggesting that increased insertional mutagenesis of the transposon is a significant factor. However it is also clear from our data that *IPB7* also exerts a detrimental effect in the absence of a supplied *piggyBac* transposon. It remains possible that the analysis of each of the seven mutations of *IPB7* may result in a transposase that provides enhanced activity in insects without any accompanying detrimental effects. At the very least the discovery of *IPB7* validates the use of yeast-based genetic screens that generate transposases with enhanced and interesting properties that may be useful in insects.

Acknowledgments

PWA and NLC are supported by PHS AI45741; NLC is a Howard Hughes Medical Institute Investigator. We thank Ms. Stephanie Russell (University of California, Riverside) for technical assistance, David O'Brochta (University of Maryland) for stimulating conversations and advice and Peter Arensburger (University of California, Riverside) for helpful discussions.

References

- Adelman ZN, Jasinskiene N, James AA. Development and applications of transgenesis in the yellow fever mosquito, *Aedes aegypti*. *Mol. Biochem. Parasitol.* 2002; 121:1–10. [PubMed: 11985858]
- Arensburger P, Kim YJ, Orsetti J, Aluvihare C, O'Brochta DA, Atkinson PW. An active transposable element, *Herves*, from the African malaria mosquito *Anopheles gambiae*. *Genetics.* 2005; 169:697–708. [PubMed: 15545643]
- Baus J, Heggestad AD, Sanz S, Fletcher BS. Hyperactive transposase mutants of the *Sleeping Beauty* transposon. *Molecular Therapy.* 2005; 12:1148–1156. [PubMed: 16150650]
- Beall EL, Mahoney MB, Rio DC. Identification and analysis of a hyperactive mutant form of *Drosophila* P-element transposase. *Genetics.* 2002; 162:217–227. [PubMed: 12242235]
- Bingham PM, Kidwell MG, Rubin GM. The molecular basis of P-M hybrid dysgenesis: the role of the P element, a P-strain-specific transposon family. *Cell.* 1982; 29:995–1004. [PubMed: 6295641]
- Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell.* 2007; 128:1089–1103. [PubMed: 17346786]
- Brennecke J, Malone CD, Aravin AA, Sachidanandam R, Stark A, Hannon GJ. An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science.* 2008; 322:1387–1392. [PubMed: 19039138]

- Cadinanos J, Bradley A. Generation of an inducible and optimized *piggyBac* transposon system. *Nucleic Acids Res.* 2007; 35:e87. [PubMed: 17576687]
- Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T. Efficient transposition of the *piggyBac* (PB) transposon in mammalian cells and mice. *Cell.* 2005; 122
- Doherty JE, Huye LE, Yusa K, Zhou L, Craig NL, Wilson MH. Hyperactive *piggyBac* gene transfer in human cells and *in vivo*. *Human Gene Therapy.* 2012; 23:311–320. [PubMed: 21992617]
- Elick TA, Bauser CA, Principe NM, Fraser MJ. PCR analysis of insertion site specificity, transcription, and structural uniformity of the Lepidopteran transposable element IFP2 in the TN-368 genome. *Genetica.* 1996; 97:127–139. [PubMed: 8901134]
- Grabundzija I, Irgang M, Mates L, Belay E, Matrai J, Gogol-Doring A, Kawakami K, Chen W, Ruiz P, Chuah MKL, VandenDriessche T, Izsvak Z, Ivics Z. Comparative analysis of transposable element vector systems in human cells. *Molecular Therapy.* 2010; 18:1200–1209. [PubMed: 20372108]
- Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O, Bendedict MQ. Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. *Insect Mol Biol.* 2001; 10:597–604. [PubMed: 11903629]
- Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, Siomi H, Siomi MC. A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in *Drosophila*. *Science.* 2007; 315:1587–1590. [PubMed: 17322028]
- Hacker U, Nystedt S, Barmchi MP, Horn C, Wimmer EA. *piggyBac*-based insertional mutagenesis in the presence of stably integrated P elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA.* 2003; 100:7720–7725. [PubMed: 12802016]
- Handler AM, McCombs SD, Fraser MJ, Saul SH. The lepidopteran transposon vector, *piggyBac*, mediates germ line transformation in the Mediterranean fruit fly. *Proc. Natl. Acad. Sci. USA.* 1998; 95:7520–7525. [PubMed: 9636182]
- Horn C, Wimmer EA. A versatile vector set for animal transgenesis. *Dev. Genes Evol.* 2000; 210:630–637. [PubMed: 11151300]
- Ivics Z, Hackett PB, Plasterk RH, Izsvak Z. Molecular reconstruction of *Sleeping Beauty*, a *Tc1*-like transposon from fish, and its transposition in human cells. *Cell.* 1997; 91:501–510. [PubMed: 9390559]
- Jasinskiene N, Coates CJ, Benedict MQ, Cornel AJ, Salazar-Rafferty C, James AA, Collins FH. Stable, transposon-mediated transformation of the yellow fever mosquito, *Aedes aegypti*, using the *Hermes* element from the house fly. *Proc. Natl. Acad. Sci. USA.* 1998; 95:3743–3747. [PubMed: 9520437]
- Jensen PA, Stuart JR, Goodpaster MP, Goodman JW, Simmons MJ. Cytochrome regulation of *P* transposable elements in *Drosophila melanogaster*: repressor polypeptides or piRNAs? *Genetics.* 2008; 179:1785–1793. [PubMed: 18579507]
- Kaminker J, Bergman C, Kronmiller B, Carlson J, Svirskas R, Patel S, Frise E, Wheeler D, Lewis S, Rubin G, Ashburner M, Celniker S. The transposable elements of the *Drosophila melanogaster* euchromatin: a genomics perspective. *Genome Biology.* 2002; 3 research0084.0081 - 0084.0020.
- Kokoza V, Ahmed A, Wimmer EA, Raikhel AS. Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the *piggyBac* transposable element vector pBac[3xP3-EGFPafm]. *Insect Biochem Mol Biol.* 2001; 31:1137–1143. [PubMed: 11583926]
- Lampe DJ, Akerley BJ, Rubin EJ, Mekalanos JJ, Robertson HM. Hyperactive transposase mutants of the *Himar1* *mariner* transposon. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96:11428–11433. [PubMed: 10500193]
- Li MA, Turner DJ, Ning Z, Yusa K, Liang Q, Eckert S, Rad L, Fitzgerald TW, Craig NL, Bradley A. Mobilization of giant *piggyBac* transposons in the mouse genome. *Nucleic Acids Res.* 2011; 39:e148. [PubMed: 21948799]
- Li X, Ewis H, Hice RH, Malani N, Parker N, Zhou L, Feschotte C, Bushman FD, Atkinson PW, Craig NL. A resurrected mammalian *hAT* transposable element and a closely related insect element are highly active in human cell culture. *Proc. Natl. Acad. Sci. USA.* 2012
- Lobo N, Li X, Fraser MJ Jr. Transposition of the *piggyBac* element in embryos of *Drosophila melanogaster*, *Aedes aegypti* and *Trichoplusia ni*. *Mol Gen Genet.* 1999; 261:803–810. [PubMed: 10394918]

- Lorenzen MD, Berghammer AJ, Brown SJ, Denell RE, Klingler M, Beeman RW. piggyBac-mediated germline transformation in the beetle *Tribolium castaneum*. *Insect Mol Biol*. 2003; 12:433–440. [PubMed: 12974948]
- Mates L, Chuah MKL, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, Grzela DP, Schmitt A, Becker K, Matrai J, Ma L, Samara-Koko E, Gysemans C, Pryputniewicz D, Miskey C, Fletcher B, VandenDriessche T, Ivics Z, Izsvak Z. Molecular evolution of a novel hyperactive *Sleeping Beauty* transposase enables robust stable gene transfer in vertebrates. *Nat. Genetics*. 2009; 41:753–761. [PubMed: 19412179]
- Mitra R, Fain-Thornton J, Craig NL. *piggyBac* can bypass DNA synthesis during cut and paste transposition. *EMBO J*. 2008; 27:1097–1109. [PubMed: 18354502]
- Munstermann, LE. Care and maintenance of *Aedes* mosquitoes colonies. Chapman and Hall; London: 1997.
- Nolan T, Bower TM, Brown AE, Crisanti A, Catteruccia F. *piggyBac*-mediated germline transformation of the malaria mosquito *Anopheles stephensi* using the red fluorescent protein dsRED as a selectable marker. *J. Biol. Chem*. 2002; 277:8759–8762. [PubMed: 11805082]
- Perera OP, Harrell IR, Handler AM. Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with a piggyBac/EGFP transposon vector is routine and highly efficient. *Insect Mol Biol*. 2002; 11:291–297. [PubMed: 12144693]
- Rubin GM, Spradling AC. Genetic transformation of *Drosophila* with transposable element vectors. *Science*. 1982; 218:348–353. [PubMed: 6289436]
- Sarkar A, Coates CJ, Whyard S, Willhoeft U, Atkinson PW, O'Brochta DA. The *Hermes* element from *Musca domestica* can transpose in four families of cyclorrhaphan flies. *Genetica*. 1997; 99:15–29. [PubMed: 9226434]
- Wang HG, Fraser MJ. TTAA serves as the target site for TFP3 lepidopteran transposon insertions in both nuclear polyhedrosis virus and *Trichoplusia ni* genomes. *Insect Mol Biol*. 1993; 1:109–116. [PubMed: 8269089]
- Wilson MH, Coates CJ, George AL Jr. piggyBac Transposon-mediated Gene Transfer in Human Cells. *Mol Ther*. 2007; 15:139–145. [PubMed: 17164785]
- Yusa K, Zhou L, Li MA, Bradley A, Craig NL. A hyperactive *piggyBac* transposase for mammalian applications. *Proc. Natl. Acad. Sci. USA*. 2011; 108:1531–1536. [PubMed: 21205896]

Highlights

The highlights of the research reported in this manuscript are:

- Proof of concept that a transposase identified in a yeast-based hyperactive screen retains activity in an insect species.
- First testing of this transposase in a metazoan.
- First demonstration that activity is also retained in a mosquito.

Fig. 1. Ratio of transposition frequencies of IPB7 and wild-type *piggyBac* transposase relative to *Hermes* transposition (y axis) from *D. melanogaster* (gray bars) and *Ae. aegypti* (black bars) transposition assays.

Fig. 2. Ovaries dissected from fertile or sterile G0 *D. melanogaster* females injected with wild-type *piggyBac* transposase or *IPB7* and vector plasmid. Ovaries from fertile (A) and sterile (C) females injected with wild-type transposase and vector. Ovaries from fertile (B) and sterile (D) females injected with *IPB7* and vector. Ovaries from a female injected only with the *IPB7* transposase and no vector plasmid (E).

Fig. 3. Ovaries dissected from an uninjected *Ae. aegypti* female (A) and one from a sterile G0 female injected with *IPB7* and vector plasmid (B). Dissections were performed two days post blood feeding.

Table 1

Transposition frequencies generated by *Hermes*, *IPB7* transposase and wild-type *piggyBac* transposase in *D. melanogaster* embryos.

Transposase	# Experiments	Donor plasmid titer	# Confirmed transpositions	Transposition frequency (Std. Dev.)	Ratio of <i>piggyBac</i> : <i>Hermes</i>
<i>Hermes</i> Control I	5	171,800	7	1.26×10^{-4} (1.76×10^{-4})	
<i>IPB7</i>	5	60,600	100	3.14×10^{-3} (3.25×10^{-3})	24.92
<i>Hermes</i> Control II	3	21,000	8	4.48×10^{-4} (3.68×10^{-4})	
<i>piggyBac</i> WT	3	25,000	28	1.14×10^{-3} (3.68×10^{-4})	2.54

Table 2

Survivorship, sterility and transgenic generation frequencies using wild-type *piggyBac* transposase and *IPB7* in *D. melanogaster*.

Transposase	Conc. (ng/ul)	Expt	# injected	#Survived injection	# Eclosed	# G0 Crosses	# Fertile	# Sterile	# Transgenic	Sterility Frequency	Transformation Frequency
Wild-type <i>PiggyBac</i>	250	I	169	104	59	59	55	4	7	6.77%	12.72%
		II	170	120	82	82	73	9	8	10.97%	10.96%
		III	152	117	68	68	64	4	4	5.88%	6.25%
Totals			491	341	209	209	192	17	19	8.13%	9.89%
Average				69.70%	61.10%	100%	91.20%	8.61%		8.80%	9.97%
Stdv				7.75%	6.33%	0%	2.70%	3.25%		2.97%	2.73%
<i>IPB7</i>	250	I	150	75	38	38	13	25	0	65.60%	0%
		II	162	86	76	72	56	16	1	20%	1.78%
		III	91	58	30	30	24	6	0	20%	0%
		IV	157	120	103	99	52	47	0	47.40%	0%
Totals			560	339	247	239	145	94	1	39.30%	0.68%
Average				60.8%	69.15%	99.12%	60.10%	84.10%		38.20%	0.45%
Stdv				11.96%	20.08%	1.94%	20.88%	74.10%		22.30%	0.009
<i>IPB7</i> Transposase Only	250	I	192	160	143	143	110	33	0	23%	0%
	50	I	210	128	119	110	74	36	0	32.72%	0%
		II	146	87	72	63	40	23	0	36.51%	0%
		III	163	99	83	72	46	26	0	36.11%	0%
Totals			519	314	274	245	160	95	0		
Average				60.42%	86.52%	88.89%	64.88%	54.22%		35.11%	
Stdv				7.32%	5.61%	3.09%	2.08%	4.85%		2.08%	
<i>IPB7</i>	25	I	151	107	63	56	46	10	0	17.8%	0%
		II	327	191	163	159	119	40	0	25.16%	0%
		III	171	127	83	78	65	13	0	16.67%	0%
Totals			649	425	309	293	230	63	0		
Average				67.86%	69.86%	93.47%	80.10%	25.12%		19.90%	
Stdv				8.34%	13.79%	4.35%	4.00%	7.41%		4.60%	

Table 3

Transposition frequencies generated by *Hermes*, *IPB7* and wild-type *piggyBac* transposase in *Ae. aegypti* embryos.

Transposase	# Experiments	Donor plasmid titer	# Confirmed transpositions	Transposition frequency (Std. Dev.)	Ratio of <i>piggyBac</i> : <i>Hermes</i>
<i>Hermes</i> Control I	3	46,800	1	1.1×10^{-4} (1.56×10^{-4})	1.15
<i>IPB7</i>	3	19,600	3	1.26×10^{-4} (9.03×10^{-5})	
<i>Hermes</i> Control II	2	132,200	6	4.25×10^{-5} (4.85×10^{-6})	0.94
<i>piggyBac</i> WT	2	155,000	7	3.97×10^{-5} (9.65×10^{-6})	

Table 4*Ae. aegypti* transformation experiments using *IPB7* transposase.

Transposase	# Experiments	# Fertile	# Sterile	# G0 Crosses	Sterility%	# Transgenic matings
<i>IPB7</i>	3	21	70	91	77%	0