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Interplasmid transposition demonstrates *piggyBac* mobility in vertebrate species

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Abstract

The *piggyBac* transposon is an extremely versatile helper-dependent vector for gene transfer and germ line transformation in a wide range of invertebrate species. Analyses of genome sequencing databases have identified *piggyBac* homologues among several sequenced animal genomes, including the human genome. In this report we demonstrate that this insect transposon is capable of transposition in primate cells and embryos of the zebrafish, *Danio rerio*. *piggyBac* mobility was demonstrated using an interplasmid transposition assay that has consistently predicted the germ line transformation capabilities of this mobile element in several other species. Both transfected COS-7 primate cells and injected zebrafish embryos supported the helper-dependent movement of tagged *piggyBac* element between plasmids in the characteristic cut-and-paste, TTAA target-site specific manner. These results validate *piggyBac* as a valuable tool for genetic analysis of vertebrates.

Introduction

The Lepidopteran-derived *piggyBac* transposon is the type element for a unique group of TTAA-targeting Class II transposable elements originally isolated as mutation-inducing insertions in baculovirus genomes (Fraser, Smith & Summers, 1983; Fraser et al., 1985; Cary et al., 1989; Wang, Fraser & Cary, 1989; see Fraser, 2001 for a review). Initial functional analyses confirmed its potential as a helper-dependent gene transfer vector (Fraser et al., 1995), and subsequent demonstrations of its effectiveness as a gene transfer vector have been performed in a number of invertebrate species including the important disease vectors *Aedes aegypti* (Kokoza et al., 2001; Lobo et al., 2002) and *Anopheles gambiae* (Grossman et al., 2001; Kim et al., 2004). Its range of utility has been expanded into non-arthropod invertebrates such as Planaria (Gonzalez-Estevez et al., 2003). As yet there has been no demonstrated mobilization of *piggyBac* in prokaryotic organisms.

This unique transposon has also become established as a highly useful transgenic vector for the model genetic system, *Drosophila melanogaster* (Handler & Harrell, 1999; Hacker et al., 2003; Horn et al., 2003; Lorenzen et al., 2003; Ryder and Russell, 2003; Bonin & Mann, 2004; Parks et al., 2004; Thibault et al., 2004). By using *piggyBac* in conjunction with *P*-element as an insertional mutagenesis tool in Drosophila the number of genes tagged in mutational screens has been significantly expanded (Parks et al., 2004; Thibault et al., 2004). In applying this vector to this invertebrate species there has been a demonstrated potential for a wide variety of useful genetic manipulations (Parks et al., 2004; Thibault et al., 2004). While this report was under review, Ding

et al. (2005) reported similar capabilities for *piggyBac* transformation and interrogation of the mouse genome.

Plasmid-based transposition assays (Coates et al., 1995, 1997; Sarkar et al., 1997; Lobo, Li & Fraser, 1999; Thibault et al., 1999; Lobo et al., 2001) have provided presumptive evidence for pursuing a given transposon as a gene transfer tool in a given species. These assays have been used to predict the capabilities for germ-line transgenesis of *Sleeping Beauty* in a variety of vertebrate systems, and the *Tol2* element in zebrafish (Ivics, Izsvák & Hackett, 1997; Kawakami et al., 1998; Izsvak, Ivics & Plasterk, 2000; Kawakami, Shima & Kawakami, 2000). In the case of the *piggyBac* element, the interplasmid transposition assay allows detection of the characteristic precise insertion and excision events (Elick, Bauser & Fraser, 1996; Elick, Lobo & Fraser, 1997; Lobo, Li & Fraser, 1999), a defining feature of the transpositional movement of this element (Fraser et al., 1995, 1996; Elick, Bauser & Fraser, 1996). In every case, demonstration of interplasmid mobilization of *piggyBac* sequences in cells or embryos of a given species has led to successful transgenic manipulation of that species (Lobo, Li & Fraser, 1999; Grossman et al., 2000, 2001; Lobo et al., 2002).

If the capabilities of precise insertion, excision, and remobilization for enhancer trapping that are exhibited by the *piggyBac* transposon in invertebrate systems such as *Drosophila* (Parks et al., 2004; Thibault et al., 2004) could be applied to model vertebrate systems such as the zebrafish, genetic analyses in these vertebrate systems could approach the wealth of capabilities developed for genetic manipulations in *D. melanogaster*. Justification for believing *piggyBac* movement is possible in vertebrate systems comes from recent analyses of genome sequencing data which identified seemingly complete as well as incomplete homologues of the *piggyBac* element among several sequenced animal genomes (Sarkar et al., 2003). In this report, we use the interplasmid transposition assay to demonstrate *piggyBac* mobilization in a helper-dependent fashion in fertilized embryos of the zebrafish, *Danio rerio*, and in the primate COS-7 cell line. This study further establishes the utility of this element for germ line transformation in many vertebrate model systems.

Methods

Preparation of plasmid DNAs

Plasmid DNAs used for transfections or microinjections were prepared using the rapid boiling procedure and were purified by CsCl gradient centrifugation. Following collection of the supercoiled fraction and extraction of the ethidium bromide with isoamyl alcohol, the DNAs were dialyzed against four changes of 4000 volumes of 0.1× SSC and stored frozen at -20°C until used. Because these plasmids were to be used for transfection of cell cultures they were handled as sterile reagents at all times. At no time were these DNAs subject to contamination with any other plasmids.

The target plasmid used in these analyses was pGDV1, a chloramphenicol resistance plasmid derived from the *Bacillus subtilis* plasmid pTZ12 (Aoki et al., 1987) by the addition of a multiple cloning site between 1970 and 2029 bp (Bron, 1995; Sarkar et al., 1997). The pB(KOα) plasmid (Thibault et al., 1999) was used as the *piggyBac* donor and was derived from a p3E1.2 plasmid derivative by insertion of a cartridge containing the kanamycin resistance gene, the ColE1 origin, and the α?peptide of β-galactosidase at a unique BglII site within the *piggyBac* open reading frame. The transposase helper was the p*hsp*Bac (formerly named pBhsΔSac) expression plasmid (Handler et al., 1998).

A stock plasmid mixture of pGDV1 (0.8 μg/μl), pB(KOα) (0.4 μg/μl) and p*hsp*Bac (0.4 μg/μl) was prepared in sterile distilled water and used for all of the COS-7 and zebrafish experiments. A separate stock plasmid mixture of pGDV1 (0.8 μg/μl) and pB(KOα) (0.4 μg/μl) was used for the transfection and injection controls.

Maintenance and transfection of COS-7 cells

African green monkey kidney cells (COS-7) were maintained by passage at 1:5 dilutions in a 37°C incubator 5% CO_2 in DMEM medium (Life Technologies) with 10% Fetal Bovine Serum (Life Technologies).

Transfections were performed using a starting cell density of 5×10^4 cells/well of a 6-well culture plate. The LipofectAMINE Plus Reagent (Life Technologies, Inc.) was combined with a total of

10 μg of the stock experimental plasmid mixture in and added to COS-7 monolayers according to the manufacturer's recommended procedure. Control transfections utilized the same reagents and 10 μg of the stock control plasmid mixture containing pGDV1 and pB(KOα) to verify both a lack of contaminating positive transposition plasmids among these reagents, and the absence of endogenous *piggyBac* transposase activity in COS-7 cells.

Microinjection of Danio rerio embryos

Fresh zebrafish eggs were collected (Friemann Centre, University of Notre Dame) and injected with DNA solution as described by Westerfield (1993). Microinjection of plasmid DNA was carried out using an agarose gel (made in Hanks Solution, Westerfield, 1993) with depressions, created by a capillary tube, as a holding place (Westerfield, 1993). The DNA solution was air-pressure-injected approximately an hour after fertilization at the 1–8 cell stage zebrafish. Injected eggs were stored at 28°C in Hanks Solution for 18 h.

Each injection set was performed independently of the others using the same plasmid DNA preparations. The experimental injections used the stock plasmid mixture of pGDV1, pB(KOα), and (p*hsp*Bac). The control injections utilized the stock control plasmid mixture containing pGDV1 and pB(KOα) to verify both a lack of contaminating positive transposition plasmids and the absence of endogenous *piggyBac* transposase activity in the zebrafish embryos.

Plasmid excision assay

A standard transposon plasmid excision assay (Lobo, Li & Fraser, 1999) was performed to determine if the COS-7 primate cells could support the first step of the cut-and-paste reaction mediated by the *piggyBac* transposase. This assay utilized the transposase helper plasmid, p*hsp*Bac, to drive excision of the *piggyBac* element from the donor plasmid, pBKOα. Equal concentrations of both plasmid DNAs were transfected at 2 and 5 μg total DNA concentration, recovered by modified Hirt (1967) extraction (Lobo, Li & Fraser, 1999; Lobo et al., 2001) at 24 h, digested with BglII, electroporated into DH10B cells, and immediately plated without recovery on LB/Amp/X-gal plates. No heat shock was used to induce expression of the transposase from the helper.

Because excision of the transposon results in removal of the lacZ gene in the plasmid, positive excision events are recovered as white colonies on LB Amp/Xgal plates. The number of recoverable donor plasmids for each experiment was estimated by electroporating a 1 μl aliquot of undigested DNA from the same Hirt extract and counting the number of blue colonies representing the donor plasmid.

Interplasmid transposition assay

Plasmid DNAs were recovered from COS-7 cells at 24 h post transfection, and from zebrafish embryos at 18 h post injection using a modified Hirt (1967) extraction (Lobo, Li & Fraser, 1999; Lobo et al., 2001), and electroporated into *E. coli* DH10B cells. Neither cells nor embryos were subjected to heat shock to induce transposase expression from the helper plasmid. Interplasmid transposition events were identified and characterized by immediate selective plating of electroporated bacteria on LB Chloramphenicol (Cam; 25 μg/ml)/Kanamycin (Kan; 50 μg/ml)/X-gal (0.025 μg/ml) plates essentially as previously described (Lobo, Li & Fraser, 1999; Lobo et al., 2001). The total amount of donor plasmid recovered was estimated by simultaneous plating of an aliquot (1%) of the transformation mix on LB Ampicillin (50 μg/ml)/X-gal (0.025 μg/ml) plates and recording the estimated number of blue colonies.

Control transfections or injections were performed using the donor and target plasmids in the absence of the helper p*hsp*Bac element insuring both that no endogenous transposase activity is evident in either COS-7 cells or zebrafish embryos. In addition, a control transformation of *E. coli* with the stock experimental plasmid mix containing all three plasmids verified the absence of background transposition events occurring in the transformed bacteria and confirmed the absence of contaminating positive transposition plasmids among all three plasmid reagents.

Analysis of transposition events

Putative interplasmid transposition products were isolated from colonies recovered from LB Cam/

Kan/X-gal plates and were directly sequenced using *piggyBac* specific primers (JF01: CCTCGATATACAGACCGATAAAAACACA-TG and JF02: GCACGCCTCAGCCGAGCT-CCAAGCGGCGAC) enabling the verification of transposition events as well as the identification of insertion sites on the pGDV1 plasmid.

Results

Excision assay to determine piggyBac mobilization in vertebrate cells

Because the *piggyBac* transposon moves using a precise cut-and-paste mechanism (Elick, Bauser & Fraser, 1996; Lobo, Li & Fraser, 1999, Lobo et al., 2001), a plasmid excision assay can be used as a predictor of *piggyBac* transposase activity. We performed a preliminary standard excision assay (Lobo et al., 2001) in COS-7 cells and zebrafish embryos using the donor and helper plasmids, pBKOα and p*hsp*Bac, respectively. The transposase providing helper plasmid, p*hsp*Bac (Handler et al., 1998) expresses the *piggyBac* ORF under the control of the *D. melanogaster hsp70* promoter, which has a demonstrated activity in vertebrate cells (Romano et al., 2001).

In both systems we recovered excision events in the presence of the p*hsp*Bac helper that were exclusively precise, characteristic of *piggyBac* transposase activity (Elick, Bauser & Fraser, 1996), while no excision events were recovered in the absence of the helper (data not shown). These results demonstrated the activity of the *piggyBac* transposase in mediating the first step of the cut-and-paste movement of the element, and confirmed the utility of the Drosophila *hsp70* promoter in these systems to drive expression of the transposase gene.

Interplasmid transposition assays in COS-7 cells

An interplasmid transposition assay (Thibault et al., 1999; Lobo et al., 2001; Figure 1) was utilized to determine if the *piggyBac* element was

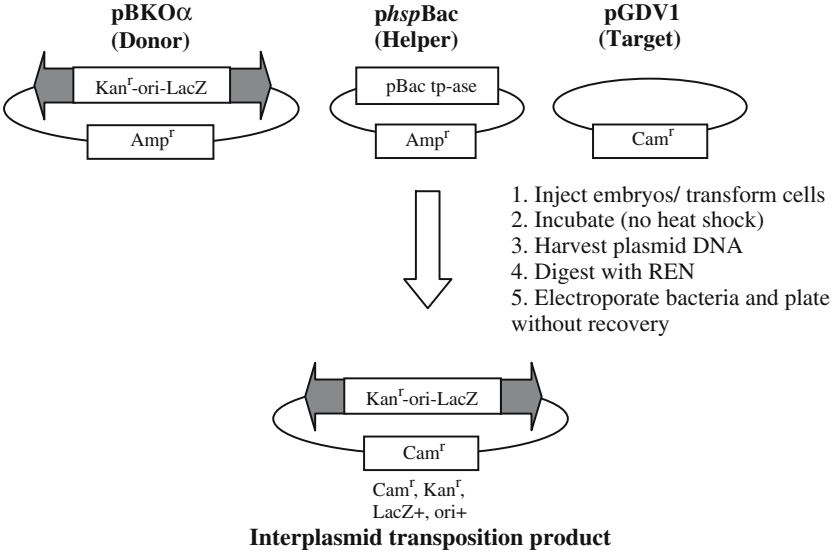

Figure 1. Interplasmid transposition assay flow diagram. A combination of 3 plasmids were introduced into cells or embryos. The donor plasmid, pB(KOα), carries a *piggyBac* element marked with the kanamycin resistance gene, ColE1 origin of replication (ori), and the α peptide of the β-galactosidase gene. The transposase providing helper plasmid, phspBac expressed the *piggyBac* ORF under the control of the *D. melanogaster* hsp70 promoter and is unable to transpose as it lacks a terminal repeat. The target *B. subtilis* plasmid, pGDV1, is incapable of replication in *E. coli*, and contains the chloramphenicol resistance gene. Transposition of the genetically tagged *piggyBac* element from the donor into the target plasmid pGDV1 with the help of the transposase provided by the helper p*hsp*Bac, results in an interplasmid transposition (IPT) product. This pGDV1 derived IPT plasmid with its acquired ColE1 ori can replicate in *E. coli* and produce blue colonies on LB/kan/cam/X-gal plates. Blue colonies that grew on LB/kan/cam/X-gal plates were grown up and plasmid DNA isolated for sequencing to confirm *piggyBac* mediated transposition.

capable of helper dependent transposition in vertebrate cells. The assay is an accurate predictor of germ-line transposition and measures the ability of the *piggyBac* element to move from a donor plasmid (pB(KOα)) into a target plasmid (pGDV1) in the presence of *piggyBac* transposase expressed from the helper plasmid (p*hsp*Bac). COS-7 cells, a vertebrate cell line derived from African green monkey kidneys (Gluzman, 1981) were co-transfected with a combination of these three plasmids. Positive transposition events were recovered from Hirt extracts of transfected COS-7 cells by plating transformed bacteria on Cam/Kan/X-gal plates. No transposition events were recovered from control transfections in the absence of the helper plasmid, demonstrating the recovered transpositions were not the result of endogenous transposase activity and the lack of contaminating positive plasmids in the donor and target plasmid preparations. A further standard control in these assays transformed all three plasmids directly into *E. coli* (Table 1). Since we had previously determined that there is no *piggyBac* mobility in these bacteria, this control effectively establishes the absence of contaminating positive plasmids among the three starting plasmid preparations.

Transposition frequencies were estimated relative to the total number of recovered donor plasmids, Amp/X-gal plates (Table 1). Fifteen interplasmid transposition events were recovered in 8 independently performed transfections, yielding a calculated cumulative interplasmid transposition frequency of 5.7×10^{-4} (Table 1).

All putative interplasmid clones were sequenced using the JF01 or JF02 outward-facing *piggyBac* specific primers (Methods), allowing identification of the insertion site on the pGDV1 plasmid. Confirmation of a transposition event was obtained by observing the characteristic duplication of a TTAA target site in the pGDV1

Table 1. Transposition of *piggyBac* in the COS-7 cell line

Cell transformed	Helper plasmid	Extraction	# wells per extraction	# Donor plasmid	# IPT events	Frequency
COS-7	p*hsp*Bac	1	6	19,800	5	5.7×10^{-4}
		2	1	800	3	
		3	1	1800	0	
		4	1	300	3	
		5	1	300	3	
		6	1	3100	1	
		7	1	100	0	
		8	1	100	0	
		Total	13	26,300	15	
Controls						
COS-7	None	1	12	~700,000	0	0
		2	12	~700,000	0	
		3	1	1000	0	
		4	1	900	0	
		Total	26	~ 1,401,000	0	
E. coli	p*hsp*Bac	13	–	~127,000	0	0

Plasmids were recovered by Hirt extraction 24 h following transfection of COS-7 cells and were transformed into *E. coli* DH10B cells. One percent of the transformed cells were plated without recovery on LB/ampicillin plates with X-Gal, and the number of blue colonies containing donor plasmids (pB(KOα)) was counted or, where necessary, estimated (# donor plasmid). The remaining cells were plated without recovery on LB plates containing Cam, Kan, and X-Gal, and blue colonies resulting from transposition events into the target plasmid (pGDV1) were counted and sequenced using the *piggyBac*-specific inverse primers JF01 and JF02 (Methods) to determine the number of precise Interplasmid Transposition events (# IPT events). The frequency of transposition into the target pGDV1 plasmid was calculated relative to the estimated number of donor plasmids recovered. Control transfections consisted of cells transfected with donor and target plasmids alone. An additional control to demonstrate a lack of bacterial mobilization and absence of contaminating transposition plasmids consisted of the three plasmids directly transformed into *E. coli* DH10B cells.

sequence on each side of the inserted transposon. Transposition events were recovered at only one of the 21 available TTAA target sites that do not result in an interruption of the chloramphenicol resistance gene (between 1169 and 1655 bp) in the pGDV1 plasmid, at base pair position 363 (Table 2), and all insertions at this site were in the same orientation.

Interplasmid transposition assays in zebrafish embryos

Following the successful demonstration of transposition in a mammalian cell line we tested *piggyBac* movement in the phylogenetically distant and experimentally valuable vertebrate model animal, *Danio rerio*. Zebrafish embryos were injected at the 1–8 cell stage with a 2:1:1 ratio of target:donor:helper plasmid ratio in a total concentration of 1.6 μg/μl. Plasmid DNA was recovered from the injected embryos 18 h post injection by Hirt extraction, electroporated into *E. coli* and assayed on selective media as described for the for COS-7 cells.

Plasmid DNAs recovered from blue Kan/Cam colonies were sequenced to verify the transpositional insertion of the KOα-marked *piggyBac* element into the pGDV1 target plasmid. A total of 10 interplasmid transposition events were recovered from five independent injection experiments yielding a combined total of 1310 injected embryos, and resulting in a cumulative interplasmid transposition frequency of 1.4×10^{-6} (Table 2). All clones possessed the characteristic TTAA tetranucleotide target site duplication flanking the inserted transposon which confirms *piggyBac*-mediated transposition. All recovered insertions occurred at base pair position 363 in the plasmid pGDV1, and all were in the same orientation. Control transformations of the combined plasmids in *E. coli* yielded no interplasmid transposition events (Table 2), confirming a lack of contaminating positives, and that the observed mobility

Table 2. Transposition of *piggyBac* in *D. rerio* embryos

Experimental	Helper plasmid	Injection	# eggs injected	# Donor plasmids	# IPT events	Frequency
Zebrafish	p*hsp*Bac	1	110	1,401,000	1	1.4×10^{-6}
		2	350	1,418,400	1	
		3	150	26,000	1	
		4	400	367,150	4	
		5	300	~3,000,000	3	
		Total	1310	~7,116,100	8	
Controls						
Zebrafish	None	1	200	~1,500,000	0	0
		2	300	~1,500,000	0	
		3	200	~1,500,000	0	
		4	200	~1,500,000	0	
		Total	900	~6,000,000	0	
E. coli	p*hsp*Bac	1	–	56,000	0	0
		2	–	71,000	0	
		Total	–	127,000	0	

Plasmids were recovered by Hirt extraction 18 h following microinjection of zebrafish embryos. One percent of the transformed cells were plated without recovery on LB/ampicillin plates with X-Gal, and the number of blue colonies containing donor plasmids (pB(KOα)) was counted or, where necessary, estimated (# donor plasmid). In several of the control injections the number of donor plasmids was estimated to be approximately the same. The remaining cells were plated without recovery on LB plates containing Cam, Kan, and X-Gal, and blue colonies resulting from transposition events into the target plasmid (pGDV1), were counted and sequenced using the *piggyBac*-specific inverse primers JF01 and JF02 (Methods) to determine the number of precise Interplasmid Transposition events (# IPT events). The frequency of transposition into the target pGDV1 plasmid was calculated relative to the counted or estimated number of donor plasmids recovered. As controls, embryos were injected with the donor and target plasmids in the absence of the helper plasmid (p*hsp*Bac), and the three plasmids were transformed directly into *E. coli* DH10B cells.

was occurring in the zebrafish embryos and not in subsequently transformed bacteria.

Interplasmid transposition assay using a deletion mutation of pGDV1

In previous analyses of a number of independent insertion sites (Li et al., 2005) we had established that there was no apparent consensus sequence configuration apart from the TTAA target site necessary for insertion of the *piggyBac* transposon. However, these interplasmid transposition results demonstrate a preferential insertion of the *piggyBac* vector at the TTAA target site at 363 bp among all alternative sites in the pGDV1 plasmid. Since we believed we had reasonably ruled out contamination as a factor, we sought an experimental verification of some alternative explanation for the observed target site preference.

We investigated the possibility that preferential insertion at a given position in the pGDV1 plasmid could result from factors other than sequence recognition by taking advantage of a spontaneous deletion mutation of pGDV1, named pGDV1Δ148, which has a deletion of sequence between 506 and 654 bp. Utilizing pGDV1Δ148 as the target plasmid in an interplasmid transposition assay in COS-7 cells we recovered 25 of 26 individual insertions at the TTAA site at position 85 bp (Table 3) instead of position 363 bp. Simultaneous control transfections in the absence of the helper plasmid yielded no transformation events. Interestingly, all these insertions were in the same orientation as those previously observed at position 363 bp. These results demonstrate that the previously observed preferential insertions at 363 bp likely result from a plasmid configuration effect rather than the affinity for a specific sequence.

Discussion

In this report we provide evidence of *piggyBac* transposition in cells of a vertebrate species. Utilizing a previously established excision and interplasmid transposition assays we demonstrate that the *piggyBac* element can mobilize in both the COS-7 vertebrate cell line and in fertilized zebrafish embryos. In both cases mobility is absent in the absence of the *piggyBac* transposase demonstrating that the intact *piggyBac* transposase is necessary for transposition and endogenous *piggyBac* homologues do not provide a detectable level of independent transposition events. As observed in a previous report (Lobo, Li & Fraser, 1999) mobility is not detected when the plasmids are passaged through *E. coli* demonstrating that the eukaryote intracellular environment is necessary for transposition and the prokaryotic intracellular environment is apparently unfavorable.

The frequency of transposition observed in zebrafish embryos is two orders of magnitude less than the frequency typically obtained with this assay in insect embryos as well as the frequency obtained in this study for interplasmid transposition in COS-7 cells, possibly reflecting the relative inefficiency of the Drosophila heat shock promoter

Table 3. Interplasmid transposition assay in COS-7 cells using pGDV1Δ148 as target

Cell transformed	Helper plasmid	Expt.	# wells in Expt.	# Donor plasmid	# IPT events	Frequency
Cos-7	p*hsp*Bac	1	1	336,800	12	3.56×10^{-5}
		2	1	420,000	14	3.33×10^{-5}
Cos-7	None	1	1	468,300	0	0
		2	1	542,800	0	0

Plasmids were recovered by Hirt extraction 24 h following transfection of COS-7 cells and the DNAs obtained were transformed into *E. coli* DH10B cells. One percent of the transformed cells were plated without recovery on LB/ampicillin plates with X-Gal, and the number of blue colonies, indicating the number of donor plasmids (pB(KOα)), was determined (# donor plasmid). The remaining cells were plated without recovery on LB plates containing Cam, Kan, and X-Gal, and the number of blue colonies, indicating transposition events into the target plasmid (pGDV1Δ148), were counted and sequenced using the *piggyBac*-specific inverse primers JF01 and JF02 (Methods) to determine the number of precise Interplasmid Transposition events (# IPT events). Control transfections consisted of cells transfected with donor and target plasmids alone. The frequency of transposition into the target pGDV1Δ148 plasmid was calculated relative to the number of donor plasmids recovered.

in expressing the transposase in zebrafish. This frequency is similar to those frequencies obtained with other transposable elements in zebrafish embryo injections. This relatively consistent reduced frequency observed among all transposons applied in injected zebrafish embryos could reflect an inherently unfavorable environment for unprotected DNA.

In these initial transposition assays of both COS-7 and zebrafish embryos all observed insertions were limited to one of the 21 recoverable TTAA insertion sites that do not interrupt the chloramphenicol gene on the target pGDV1 plasmid, and all were in the same orientation. This apparent target site and orientation preference is not without precedent. Our own previous studies had noted an apparent preference for insertion at one or two target sites within pGDV1 in both *D. melanogaster* and *Ae. aegypti* (Lobo, Li & Fraser, 1999). Interestingly enough, in these insect embryos insertions were limited to positions 363 and 491, with position 363 being the most favored site in Drosophila. Further, the insertions recovered at position 393 in all insect species previously tested happened to be in the same orientation (Lobo, Li & Fraser, 1999) corresponding with the orientation observed in these studies. In contrast, insertions at several alternative sites were recovered from the lepidopterans *Trichoplusia ni* (Lobo, Li & Fraser, 1999) and *Pectinophora gossypiella* (Thibault et al., 1999), with no apparent orientation preferences at those alternative sites.

We consider contamination to be ruled out for both our previous (Lobo, Li & Fraser, 1999) and current results for several reasons. First, the plasmid mixtures for each experimental series, whether COS-7 or zebrafish, were also used in several control transfections, injections, or transformations that demonstrated both a lack of mobility due to resident *piggyBac* homologues known to be present in each species genome (Sarkar et al., 2003) and a lack of contaminating positive plasmids in all the reagents used, including the pGDV1, helper, and donor plasmids. If contamination of reagents were a problem we would have recovered positive insertion events in one or more of these controls. Likewise, if starting plasmids were contaminated in some manner we would also have recovered transposition events in one or more of the controls, and at the very least, in the direct transformations of the plasmids in *E. coli*. Second, each of the transfection or injection experiments were carried out independently and at separate times, with all Hirt extraction and bacterial transformation reagents having been freshly prepared. In addition, the electroporation competent *E. coli* DH10B were purchased and not prepared in our lab, and therefore would be free of contamination.

We also note that while initial experiments in some species indicated a species-dependent preference for certain TTAA sites, subsequent analyses proved these apparent preferences were not strict. We therefore believe this apparent target site preference to be the result of limited sampling size.

We interpret the evidence to indicate that the pGDV1 plasmid may present a configuration within some cells that favors insertion at a particular TTAA target site, and possibly a particular orientation at that site. This interpretation is further supported by a second interplasmid transposition assay performed in COS-7 cells using the pGDV1Δ148 deletion plasmid which removed 148 bp of sequence between 506 and 654 of the pGDV1. Using this plasmid as the target we were unable to recover any insertions at position 393, but did recover 25 of 26 individual insertions at position 85, all in same orientation. Interestingly, this orientation corresponded to the orientations observed for all position 85 insertions recovered in previous insect embryo assays (Lobo, Li & Fraser, 1999), and for those position 393 insertions recovered in the present COS-7 and zebrafish assays. We do not have an explanation for this orientation preference at this time.

The most successful transgenesis system currently available for vertebrates is the pantropic retrovirus vector (Lin et al., 1994; Gaiano et al., 1996a, b; Amsterdam & Hopkins, 1999). Pantropic retrovirus vectors provide a significant improvement in the identification of mutated genes compared to chemical mutagenesis strategies by tagging genes associated with a phenotypic alteration (Gaiano et al., 1996b; Amsterdam & Hopkins, 1999). In addition, the retrovirus approach potentially allows reinsertion of mutated genes for analysis of function, limited promoter or enhancer trapping, or directed gene knockouts using RNAi approaches (e.g. Korn et al., 1992; Sablitzky et al., 1993; Xiong, Leahy & Stuhlmann, 1999). However, retrovirus vectors lack some significant capabilities of an ideal transgenesis vector such as

remobilization following insertion and a carrying capacity greater than 10 kb. They are also difficult to produce and present a biohazard to laboratory personnel (Linney et al., 1999; BD Biosciences/ Clontech manual). A suitable transposon vector could provide a desirable alternative to retroviruses in developing functional genomics of vertebrate systems.

An ideal transposon vector should facilitate not only the identification of tagged genes through frequent and mechanistically predictable insertion and excision, but should also allow defined regulation of movement permitting the development of enhancer and suppressor trapping capabilities. These manipulations are essential for full development of functional genomics in model vertebrate systems.

Application of *piggyBac* in the *D. melanogaster* model system has validated its capabilities as a versatile genetic transformation vector with many of the most desirable transposon vector capabilities (Parks et al., 2004; Thibault et al., 2004). While this manuscript was in review, similar capabilities were reported for *piggyBac* in the transformation of the mouse genome and human cell cultures (Ding et al., 2005). Our results further demonstrate that this insect transposon can move in widely divergent vertebrate systems.

The mobility of *piggyBac* in a variety of model systems and target organisms permits the testing, verification, and perfection of strategies in easily manipulated models, and application of those proven strategies to other, less tractable models. Based upon these observations and the recent publication of Ding et al. (2005) there is ample justification to expect that the *piggyBac* element would mediate germ line transformation in many higher vertebrates, extending its effective range throughout the animal kingdom.

While the extension of *piggyBac* mobility into vertebrate systems is exciting from a genetic and functional genomic standpoint, this report supports and enhances concerns for using this transposon in particular, and virtually any transposon with vertebrate homologues, for applied genetic engineering of agricultural or medical pest species. In this regard, the recent report by Handler, Zimowski and Horn (2004) of post-transformation inactivation of a *piggyBac* transposon could provide a certain amount of security against possible escape of engineered genes from genetically modified target species. However, the use of autonomously mobile *piggyBac* elements should be ruled out due to its apparently unlimited mobility characteristics.

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