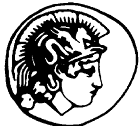

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Use of the *piggyBac* transposon for germ-line transformation of insects

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Abstract

Germ-line transformation of insects is now possible with four independent transposable element vector systems. Among these, the TTAA-insertion site specific transposon, *piggyBac*, discovered in *Trichoplusia ni*, is one of the most widely used. Transformations have been achieved in a wide variety of dipterans, lepidopterans, and a coleopteran, and for many species, *piggyBac* transposition was first tested by plasmid-based mobility assays in cell lines and embryos. All plasmid and genomic insertions are consistent with the duplication of a TTAA insertion site, and most germ-line integrations appear to be stable, though this is largely based on stable marker phenotypes. Of the vector systems presently in use for non-drosophilids, *piggyBac* is the only one not currently associated with a superfamily of transposable elements, though other elements exist that share its TTAA insertion site specificity. While functional *piggyBac* elements have only been isolated from *T. ni*, nearly identical elements have been discovered in a dipteran species, *Bactrocera dorsalis*, and closely related elements exist in another moth species, *Spodoptera frugiperda*. It appears that *piggyBac* has recently traversed insect orders by horizontal transmission, possibly mediated by a baculovirus or other viral system. This interspecies movement has important implications for the practical use of *piggyBac* to create transgenic insect strains for field release. Published by Elsevier Science Ltd.

Keywords: *piggyBac* transposon; Transposable elements; Insect transgenesis; Germline transformation; Fluorescent protein marker; Horizontal transmission

1. Introduction

The first attempts to genetically transform non-drosophilid insects with transposon-based vector systems utilized those that had already become routine for *Drosophila*. These included the *P* system (Rubin and Spradling, 1982), and then *hobo* when it became available several years later (Blackman et al., 1989). The impetus to test these systems came primarily from the fact that they were functional for at least one insect species, though failed attempts at transformation in a variety of other insects suggested that their function might be restricted in non-drosophilids (see Handler, 2000, 2001). Mobility assays that could test transposon function in cell lines and embryos, indeed, proved that *P* was nonfunctional outside the Drosophilidae (Handler et al., 1993), and that *hobo* function was limited (see O'Brochta and Atkinson, 1996). These studies stimulated the search for new transposable element systems having less restricted mobility properties, and also provided the tools to rapidly assess whether they had the potential for vector function beyond the species in which they already existed. Several of these systems are discussed in this issue, and one of them is the *piggyBac* transposon originally discovered in the cabbage looper moth, *Trichoplusia ni* (Fraser et al., 1983). The *piggyBac* element (then known as IFP2) was actually discovered about the time that *P* was first being used as a vector in *Drosophila*, but its use for insect transformation awaited a determination of its function by mobility assays. More than a dozen years after its initial discovery, a *piggyBac* vector was used to transform the Mediterranean fruit fly (Handler et al., 1998), and shortly afterwards it was used to transform a variety of insect species spanning three orders. Recent information, however, regarding the function and distribution of *piggyBac* has great relevance to how *piggyBac* is used for both basic and applied studies.

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2. Discovery of *piggyBac* and other TTAA-specific elements

Previous to the discovery of *piggyBac*, Few Polyhedra (FP) plaque morphology mutations in baculoviruses were already known to result from insertion of mobile genetic elements from host cell genomes (Miller and Miller, 1982). The research of Fraser and colleagues (see Fraser, 2000) subsequently resulted in the isolation of several transposons within the FP locus of AcNPV and GmNPV, and several of these shared specificity for insertion in the tetranucleotide TTAA site. Among these were TFP3 (*tagalong*) elements found in AcNPV and GmNPV, and IFP2 (*piggyBac*) elements found in AcNPV, after passage through the *T. ni* cell line, TN-368 (Fraser et al., 1983, 1985). Although *tagalong* elements have imperfect inverted terminal repeat sequences that have the ability to transpose, autonomous elements have not been discovered and none have transposase coding regions (Wang et al., 1989; Wang and Fraser, 1993). In contrast, the *piggyBac* elements were found to be identical having a length of 2472 kb with 13 bp perfect inverted terminal repeats and 19 bp subterminal repeats located 31 bp from the 5′ ITR and 3 bp from the 3′ ITR (Cary et al., 1989) (see Fig. 1). In between the subterminal repeats exists a transcriptional unit of 2.1 kb having a single reading frame of 1.8 kb. This encodes a putative transposase with a predicted molecular mass of 64 kDa (Elick et al., 1996).

Transposase function and the mobility properties of *piggyBac* were first determined by viral and plasmid transposition and excision assays. Initial assays in the SF21AE cell line used *piggyBac* marked with *polh/lacZ* as an indicator mobilized by transposase within the plasmid p3E1.2. These and subsequent assays provided the first evidence for p3E1.2 encoding a functional transposase; they defined a TTAA insertion-site specificity, the precise nature of *piggyBac* transposition, and provided initial data for *piggyBac* mobilization in other lepidopterans (Fraser et al., 1995). These data helped assess the potential for the use of *piggyBac* as a transformation vector, which was critical given the demonstrated lack of function of *P* in non-drosophilids. More direct evidence for *piggyBac* function, discussed below, was provided by plasmid-based transposition assays in a variety of insect species. Of particular importance to the use of *piggyBac* in insertional mutagenesis studies for functional genomics is the precise nature of its cut-and-paste transposition mechanism that is discussed in detail by Fraser (2000).

3. *piggyBac* transformation

The initial results from mobility assays showing *piggyBac* function in insect species other than *T. ni* were available about the time that evidence indicated that the *P* vector would not be functional in non-drosophilids without substantial modification. *Minos* transformation of the Mediterranean fruit fly, *Ceratitis capitata*, white eye strain was just reported (Loukeris et al., 1995), and this was made possible by the isolation of the *white* cDNA marker (Zwiebel et al., 1995). Availability of this marker made the similar testing of *piggyBac* in the medfly a possibility. Since the minimal sequence requirements for transposase function and *piggyBac* movement were uncertain, the first *piggyBac* vector and helper were conservative constructs. The first vector had the 3.6 kb hsp-*white* cDNA inserted into the unique *Hpa*I site in the middle of *piggyBac* within p3E1.2. This disrupted the transposase reading frame, but did not delete any sequence. Construction of the helper took advantage of *Sac*I in the pUC18 multiple cloning site of p3E1.2 and a unique *Sac*I site in a region 5′ to the transposase transcriptional start to create a deletion of the 5′ ITR. Although there is some debate as to whether this deletion includes the RNA polymerase II site (Cary et al., 1989), the first germ-line transformation experiment with this helper in medfly did indeed result in three independent *piggyBac*-mediated integrations in one G_0 fly at a general frequency of 5% per fertile G_0 (Handler et al., 1998). This experiment with a *piggyBac*-regulated helper was repeated with five additional G_1 lines isolated, but at approximately the same frequency. Although transformation frequencies were low relative to other systems using hsp-regulated helpers, it was nevertheless notable that a lepidopteran transposon vector system had autonomous function in a dipteran species. To this point, all other insect transformations were accomplished in dipterans with a transposon vector originally isolated from another dipteran, and only in *Drosophila* were transformations achieved with self-regulated helpers. This was encouraging for the use of *piggyBac* in a wider range of insect species (see Table 1).

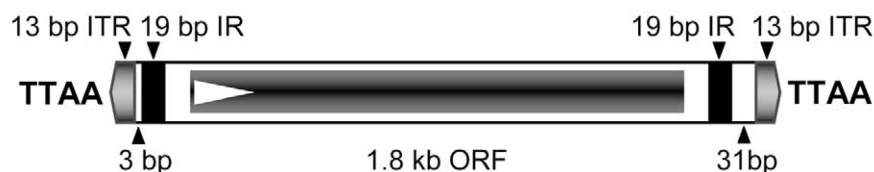

Fig. 1. Diagram of the 2.47 kb *piggyBac* element (not to scale) showing positions of the inverted terminal repeat (ITR) sequences, sub-terminal inverted repeat (IR) sequences, 1.8 kb open reading frame (ORF), and duplicated TTAA insertion site.

Table 1
Species transformed with *piggyBac* and the markers used for selection

Host species	Marker	Reference
Aedes aegypti	Dm *cinnabar*	Kokoza et al. (2001); Lobo et al. (2002)
Anastrepha suspensa	PUbnlsEGFP	Handler and Harrell (2001a)
	PUbnls-DsRed1	A. Handler and R. Harrell (unpub.)
Anopheles albimanus	PUbnlsEGFP	Perera et al. (2002)
Anopheles gambiae	hr5-ie1-EGFP	Grossman et al. (2001)
Anopheles stephensi	actin 5C-dsRED	Nolan et al. (2002)
	3xP3-EGFP	Ito et al. (2002)
Bactrocera dorsalis	Cc *white*	Handler and McCombs (2000)
	PUbnlsEGFP	A. Handler and S. McCombs (unpub.)
Bombyx mori	*Bm* actin A3-EGFP	Tamura et al. (2000)
	3xP3-EGFP	Thomas et al. (2002)
Ceratitis capitata	Cc *white*	Handler et al. (1998)
	PUbnlsEGFP	A. Handler and S. McCombs (unpub.)
	PUbnls-DsRed1	A. Handler, R. Krasteva, G. Franz, and A. Robinson (unpub.)
Drosophila melanogaster	*white*; *white*/PUbnlsEGFP	Handler and Harrell (1999)
	3xP3-EGFP	Berghammer et al. (1999); Horn et al. (2002)
	3xP3-ECFP, 3xP3-EYFP	Horn and Wimmer (2000)
	PUb-DsRed1	Handler and Harrell (2001a,b)
	3xP3-DsRed	Horn et al. (2002)
Lucilia cuprina	PUbnlsEGFP	Heinrich et al. (2002)
Musca domestica	3x3P-EGFP	Hediger et al. (2001)
Pectinophora gossypiella	Bm actin A3-EGFP	Peloquin et al. (2000)
Tribolium castaneum	3xP3-EGFP	Berghammer et al. (1999)
	3xP3-ECFP, 3xP3-EYFP, 3xP3-DsRed1	M. Klingler (unpub.)
	Tc *vermilion*	M. Lorenzen and R. Beeman (unpub.)

To determine if *piggyBac* could mediate transformation in another dipteran, the second *piggyBac* transformation was performed in *D. melanogaster* using, essentially, the same vector and helper as used in medfly, except that the Drosophila mini-*white* marker was used (Handler and Harrell, 1999). Similar to medfly, transformant lines were isolated at a similar frequency of 1–3%. Beyond extending the range of *piggyBac* vector function, transformation in *Drosophila* provided a convenient system to rapidly test helper and vector modifications to enhance their efficiency. The first modification was to insert the *hsp70* promoter into the p3E1.2 *Sac*I deletion to create a new helper having the *hsp* promoter upstream to the *piggyBac* promoter. Consistent with other systems using an *hsp*-helper, transformation frequencies with the mini-*white* vector rose dramatically to approximately 26%.

4. Testing of transformant marker systems with *piggyBac*

These successful *piggyBac* transformations, and the development of a highly efficient transposase helper plasmid encouraged others interested in this methodology to test the system in a variety of other insect species. However, while the *Drosophila* and medfly transformations could take advantage of an eye color mutant-rescue selection system, only a few other non-drosophilid species could be similarly transformed in the absence of mutant strains and cloned wild type alleles that can be used as a marker. For example, a *white eye* strain of the Oriental fruit fly, *Bactrocera dorsalis* (McCombs and Saul, 1992) was used as a host for transformation with the *piggyBac/*medfly *white*$^{+}$ vector (Handler and McCombs, 2000), and *Tribolium castaneum* has been transformed with *piggyBac* marked with the Tribolium *vermilion* gene (R. Beeman, personal communication). However, for testing gene transfer in more diverse insect species it became apparent that more generally applicable dominant-acting markers would be needed.

The jellyfish green fluorescent protein (GFP) (Chalfie et al., 1994) had already been used as a reporter in *Drosophila* (Wang and Hazelrigg, 1994; Davis et al., 1995), and Higgs et al. (1996) showed that GFP could be used as a marker for Sindbis viral infection in Culex. Thus, several laboratories began testing GFP regulated by a variety of promoters as a marker for gene transfer. We were interested in having GFP regulated so that it would be expressed from numerous tissues throughout development, using a promoter that was highly conserved to increase the possibility of wide applicability. A promoter previously tested in *Drosophila* that fulfilled these requirements was from the polyubiquitin gene (Davis et al., 1995). Ubiquitin sequence is highly conserved from yeast to humans (Lee et al., 1988), though it was

unknown if function was conserved as well. The *Drosophila* polyubiquitin promoter and SV40 nuclear localizing sequence were linked to the enhanced GFP (EGFP) and inserted into a *Bgl*II-*Hpa*I 768-bp deletion of p3E1.2 to create pB[PUbnlsEGFP] (Handler and Harrell, 1999). For initial tests, the mini-*white* gene was also inserted into the vector and pB[PUBnlsEGFP,*Dmw*] was transformed into the *D. melanogaster* *w*[m] strain using the phspBac helper. While *white*$^{+}$ was used as a control, it was possible to screen for GFP expression in larvae and pupae before adult emergence. Indeed, all the GFP lines were selected in G_1 larvae, but it was surprising to find that less than half the fluorescent G_1 flies had detectable eye pigmentation. Since the EGFP and *white*$^{+}$ markers were contiguous within the vector, this indicated that GFP expression was less susceptible to chromosomal position effects than *white*, which is very encouraging for GFP being an effective marker. The same conclusion was reached by Wimmer and colleagues testing GFP and GFP variants under the control of the Pax-6-activated promoter (Horn and Wimmer, 2000).

While the PUbnlsEGFP marker was functional in *Drosophila*, indicating that the EGFP optimized for mammalian expression was effective in insects, it still remained to be determined if the polyubiquitin promoter would function in non-drosophilids. A useful system to test this was the Caribbean fruit fly, *Anastrepha suspensa*, for which no documented visible mutations exist that could be used for mutant-rescue selection. Transformation of caribfly with the pB[PUbnlsEGFP] vector and phspBac helper resulted in four independent transgenic lines having one to four integrations, with integration sites in two sublines isolated by inverse PCR and sequenced, revealing duplication of TTAA insertion sites (Handler and Harrell, 2001a). The same vector was subsequently used to transform the medfly, oriental fruit fly (A.M. Handler and S.D. McCombs, unpublished), and the South American malaria vector, *Anopheles albimanus* (Perera et al., 2002), and the PUbnlsEGFP marker is now used for routine transformation in these species. In the caribfly study, a quantitative spectrofluorometric assay for GFP was developed. Although PUbnlsEGFP was less susceptible to position effects than *white* in *Drosophila* transformants, spectrofluorometric analysis showed that PUbnlsEGFP expression varied several-fold in different caribfly transformants having the same number of integrations (Handler and Harrell, 2001a).

Recently, a new red fluorescent protein, DsRed1 (Matz et al., 1999), was placed under polyubiquitin promoter regulation and was used to efficiently transform *Drosophila* (Handler and Harrell, 2001b), as well as *A. suspensa* (A. Handler and R. Harrell, unpublished) and *C. capitata* (A. Handler, R. Krasteva, G. Franz, and A. Robinson, unpublished). Notably, DsRed1 fluorescense was typically more intense than EGFP, and could be distinguished from GFP fluorescense when co-expressed. This is encouraging for the use of multiple markers in transgenic strains including insertional mutagenesis experiments. DsRed has also been used to select *piggyBac* transformants in *An. stephensi* (Nolan et al., 2002) and *Tribolium castaneum* (M. Klingler, unpublished).

Efforts to make initial transformant screening simpler have succeeded using the PUb-DsRed1 marker along with a *hsp70*-regulated neomycin phosphotransferase II (NPTII) gene allowing resistance to the neomycin analog, G418 (Geneticin®). While use of NPTII by itself for selection has proven to be inconsistent, it can be highly effective for screening G_1 transformants en masse when used with a visible marker that can verify transformation and transformants during breeding. We tested a dual-marked *piggyBac* vector, pB[RFP,hsneo], in *Drosophila* and from 257 G_0 adults intermated in 14 groups, 860 G_1 progeny were resistant to 250 μg/ml G418 and expressed DsRed. While some non-transformed flies survived the low stringency selection (see Steller and Pirrotta, 1985), they were easily identified by the lack of fluorescense. The G418 resistance level of the transformed flies is now being tested to develop a more stringent selection, and the dual transformant selection system will be tested in other species where it may greatly enhance the ability to screen for transformants.

Another GFP marker, regulated by the *Bombyx mori* actin A3 promoter, was used to select *piggyBac* transformants in two Bombyx strains (Tamura et al., 2000), and was also used to transform the pink bollworm, *Pectinophora gossypiella* (Peloquin et al., 2000). Both experiments yielded transformation frequencies of approximately 1–3%, though the silkmoth experiment used a transposase helper regulated by the same constitutive actin A3 promoter, while the pink bollworm experiment used the phspBac helper.

One of the more widely used *piggyBac* vectors is also marked with EGFP, but regulated by an artificial (3xP3) promoter that is activated by the phylogenetically conserved transcription factor, Pax-6 (Berghammer et al., 1999; Horn and Wimmer, 2000; see Horn et al., 2002. This was initially tested in *D. melanogaster*, but was also found to be effective in a coleopteran, *Tribolium castaneum*. This marker and its derivatives, having several other fluorescent protein markers under Pax-6 regulation, have been used to select tranformants in a variety of fly and moth species (see Horn et al., 2002). Among these were the dipterans *An. stephensi* (Ito et al., 2002) and *Musca domestica*, which was transformed at a frequency of about 17% (Hediger et al., 2001). Transformation of *B. mori* was repeated with the 3xP3-EGFP vector, and while the resultant frequency was less than the original experiments, the ability to select transformants in a liquid media previous to larval hatching made screening much more efficient (Thomas et al., 2002).

The Tribolium transformation was especially significant since this was the first germ-line transformation

reported in a coleopteran, and the first in a species outside of flies and moths. However, the initial report stated a relatively high frequency of integrations, but these were based on 3xP3-EGFP marker expression and were not molecularly characterized (Berghammer et al., 1999). Some of the insertion sites have since been sequenced, and all have the canonical duplicated TTAA insertion site (R. Beeman, personal communication). Tribolium was later transformed using 3xP3/EGFP with a *vermilion* marker, where there was a high correlation between somatic *v* marker expression in G_0 adults and G_1 germ-line transformants. Curiously not all integrations were precise with some appearing as concatemers and vector rearrangements (M. Lorenzen and R. Beeman, personal communication).

5. Testing *piggyBac* function by mobility assays as a prelude to gene transfer

Optimism that the first *piggyBac* transformations in medfly and *Drosophila* would be successful were based upon evidence for *piggyBac* transposase function and terminal sequence mobility provided by excision assays in lepidopteran cell lines, and preliminary data from embryonic assays in flies and moths (see Fraser, 2000). The testing of *piggyBac* transformation in several other dipteran and lepidopteran species was, however, preceded by excision and transposition assays that were originally developed to test *P* and *hAT* transposons (O'Brochta and Handler, 1988; O'Brochta et al., 1994). Plasmid excision and interplasmid transposition of *piggyBac*, dependent on the phspBac helper, was demonstrated in the pink bollworn, *Pectinophora gossypiella* (Thibault et al., 1999) with germ-line transformation of the species achieved subsequently (Peloquin et al., 2000). Insertion site specificity for TTAA was shown for both plasmid and genomic transposition. Use of the same transposition assays in *D. melanogaster*, *Aedes aegypti*, and *T. ni* yielded similar results, but the dependence of transposition in *T. ni* on the presence of phspBac helper was surprising since the species harbors several functional elements (Lobo et al., 1999). It could be argued that the assay lacks sufficient sensitivity to detect transposase from a few genomic copies since relatively low transposition frequencies occur when millions of plasmid-encoded helper genes are injected. Genomic repression similar to that exhibited by the *P* element in *Drosophila* has also been suggested (Fraser, 2000), though *P* repression is thought to result from multiple defective elements. Transposition was also demonstrated in other *Aedes* species (Lobo et al., 2001), and transformation of *Ae. aegypti* with *piggyBac* was reported subsequently using both the *cinnabar* (kh^{w}) (Lobo et al., 2002) and 3xP3-EGFP markers (Kokoza et al., 2001). Transposase-dependent *piggyBac* transposition was also demonstrated in both a cell line and embryos of *Anopheles gambiae* (Grossman et al., 2000), and transformation has since been achieved with a GFP-marked vector in this highly important species (Grossman et al., 2001). Nevertheless, only a single integration has been verified thus far, yielding a frequency close to 1%, which is in contrast to the 20–40% frequencies observed in *Anopheles albimanus* transformed with pB[PUbnlsEGFP] (Perera et al., 2002). The *An. gambiae* experiment used a GFP marker regulated by the baculovirus IE1 promoter, and frequencies may increase using other promoters and improved methods for DNA delivery.

Most recently, *piggyBac* transposition assays and germ-line transformation have been repeated in *D. melanogaster* (Li et al., 2001a) and performed for the first time in the Australian sheep blowfly, *Lucilia cuprina* (Heinrich et al., 2002), in which several helpers having transposase under different promoter regulation were tested and compared. In *Drosophila*, an *hsp70*-regulated transposase yielded the highest transposition frequency with or without heat shock, while a constitutive *α1-tubulin* promoter was more effective for germ-line transformation. However, in *Lucilia* the *hsp70* helper was most effective for both plasmid and germ-line transpositions, and the *α-tub* promoter failed to yield transformants. For these tests, as well as others, it is important to realize that the many variables involved in transformation make it difficult to conclusively determine reliable frequencies when relatively few fertile G_0 adults are tested or when only a few independent experiments are reported. For example, the first use of *hsp70*-helper in *Drosophila* yielded a 26% frequency (Handler and Harrell, 1999), while the more recent experiment yielded a frequency of less than 3% (Li et al., 2001a). While initial experiments using phspBac in medfly also yielded relatively low transformation frequencies, many repetitions using a variety of vectors with sizes up to 14 kb now routinely result in frequencies of 15–25% (A.M. Handler, unpublished). Initial transformation frequencies for any vector system in a new host insect should probably be regarded cautiously until verified by repetition by different laboratories using vectors of varying size.

Evaluating vector efficiency by transposition assays is also not necessarily straightforward. While these assays are highly valuable for qualitatively determining vector function in a specific insect host embryo, their predictive value for assessing relative genomic transposition frequencies has been inconsistent. This may not be unexpected, since vectors shown to be highly active in transient assays may result in greater organismal death when integrating into lethal loci within the germ-line, decreasing the frequency of surviving transformants. Nevertheless, transposition assays have yielded important information in terms of target site preference, although the significance of preferred *piggyBac* targets is still speculative. Preferences among the 21 TTAA target sites in

the pGDV1 target plasmid have been demonstrated in all the transposition assays reported, and in *Drosophila* there is a bias for those having A or T nucleotides at positions −3, −1, +1, and +3 relative to TTAA (Lobo et al., 1999; Li et al., 2001a). Mobility assays also provide a rapid means of testing sequence requirements for vector mobility, which allow modifications for more efficient vector function. For example, excision and transposition assays performed in *T. ni* embryos showed that the *piggyBac* inverted terminal repeat and sub-terminal repeat sequences were sufficient for transposition, but that a spacer region between the ITRs of greater than 40 bp is necessary for optimal transposition from a plasmid (Li et al., 2001b). Since vector mobility is known to be negatively affected by increasing size, this information should allow minimal vectors to be created that retain optimal function.

6. Phylogenetic distribution of *piggyBac*

6.1. piggyBac in Bactrocera

An unexpected finding from the Southern analysis of *B. dorsalis* transformants was that *piggyBac* hybridizing elements exist in the host strain (Handler and McCombs, 2000). These were identified as non-vector fragments in the transformant and control non-transformant samples. The major fragment in all samples digested with *Nsi*I was about 2 kb which corresponds to the internal 2.0 kb *Nsi*I fragment in *piggyBac* within p3E1.2. The relative intensity of the band suggested that it represents several copies of *piggyBac*. Three to four additional larger bands were also detected, indicating that other *piggyBac* elements exist having either DNA insertions, duplications, or possibly internal deletions that removed one of the restriction sites. In total, a conservative estimate of eight to ten elements per haploid genome was deduced, however one of the *Nsi*I fragments having a size of 4 kb was clearly polymorphic. It was not detected in the *we* host strain or a wild strain, but it appeared in two independent transgenic lines and also appeared in several *B. dorsalis* mutant strains. Finding the source of this element is certainly of interest, with possible explanations including an unstable element (which infers a source of functional transposase), or unequal recombination between contiguous duplicated sequences resulting in segregrants having duplicated or deleted sequence.

To determine the nature of these elements PCR was performed in several *B. dorsalis* strains using conserved primers spanning 1.5 kb of the 2.1 kb transposase coding region. Genomic clones from a wild *B. dorsalis* EMBL3 λ library were also isolated and sequenced along with proximal genomic sequences. Several PCR sequences and the corresponding sequence from the genomic clones all maintained a high level of identity of greater than 98% to the functional 3E1 *piggyBac* element from *T. ni*, although discrete nucleotide deletions caused frame shifts in all the sequences. Only in a sequence from the *white eye* strain did a single 6 bp deletion allow maintenance of a single reading frame over the region. Notably, while the genomic clones are probably nonfunctional, the terminal and sub-terminal inverted repeat sequences were maintained and the genomic insertion site was a TTAA duplication. Existence of functional genomic *piggyBac* elements in *B. dorsalis* may be assessed most straightforwardly by mobility assays performed without exogenous helper. Nevertheless, some of the transgenic lines have remained stable for over 20 generations based on phenotype and hybridization patterns, which is consistent with a lack of functional elements.

B. dorsalis is part of a species complex comprising more than 40 sibling species, and thus it is of some interest to determine the range of *piggyBac*s present among these species as well as other related bactrocerids. Control hybridizations to the melonfly, *B. cucurbitae*, and the Mediteranean fruit fly, *Ceratitis capitata*, did not yield discernible hybridization (Handler and McCombs, 2000), but PCR on several *B. dorsalis* strains and other *Bactrocera* species did yield related sequences having greater than 95% identity to the 3E1 *piggyBac,* and about 92% identity among all the sequences (A.M. Handler, unpublished). Thus a closely related *piggyBac* element exists among a wide range of *B. dorsalis* complex species and possibly other bactrocerids.

6.2. piggyBac in other insects

Given the evolutionary distance between *T. ni* and the Bactrocera, it is quite likely that the transposon moved among these species by recent horizontal transmission. Given the separation of their geographical habitats, this movement may have been mediated by one or more intermediary species as well. To assess the presence of *piggyBac* in a wide range of insect species, PCR was performed on more than 50 species including moths, flies, beetles, weevils, and ants (A.M. Handler, unpublished). Although PCR products having the expected size were observed in many species, most were not abundant and other PCR products were also usually evident. To more conclusively determine the presence of *piggyBac*, most of these species were subjected to Southern analysis by digesting genomic DNA with *Nsi*I and hybridizing with the 1.4 kb Nsi-Hpa probe from the *piggyBac* 5′-end (Fig. 2). This should result in a 2 kb band in *piggyBac*, and this was the only fragment detected in the *T. ni* sample. The *B. dorsalis* sample yielded the 2 kb fragment and other fragments as noted previously. Hybridization to small size fragments was detected in several species including *Ephestia kühniella*, with some

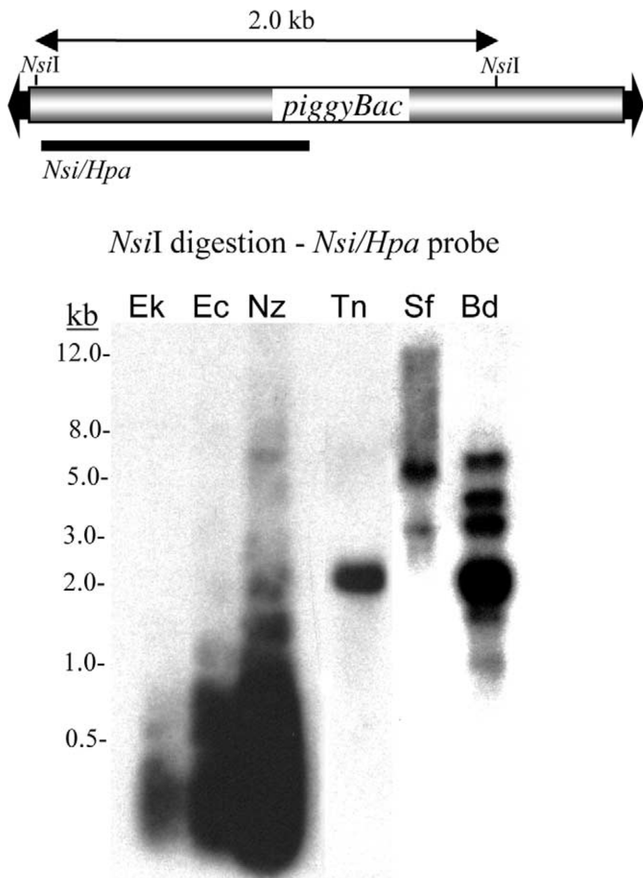

Fig. 2. Southern DNA hybridization analysis of genomic DNA from several insect species. DNA was digested with *Nsi*I restriction enzyme, separated by agarose gel electrophoresis and blotted to nylon membrane. The blot was hybridized to the 5′*piggyBac* 1.4 kb *Nsi*I-*Hpa*I restriction fragment labeled with radioisotope. See Handler et al. (1998) for detailed methods. Above the blot is a diagram (not to scale) of *piggyBac* showing positions of the *Nsi*I sites and the *Nsi/Hpa* probe. DNA came from the species *Ephestia kühniella* (Ek), *Ephestia cautella* (Ec), *Nezara viridula* (Nz), *Trichoplusia ni* (Tn), *Spodoptera frugiperda* (Sf), and *Bactrocera dorsalis* (Bd).

larger fragments detected in *E. cautella* and *Nezara viridula*. However, these hybridization patterns could not be repeated and further analysis is necessary to determine whether *piggyBac* or related elements actually exist in these species. However, *piggyBac* hybridization was also detected in *Spodoptera frugiperda*, but limited to fragments having sizes of approximately 3 kb and larger, and this has been reaffirmed in two independent hybridizations. While hybridization indicates the presence of elements closely related to *piggyBac*, the absence of 2 kb fragments indicates that they have not maintained their structural integrity and are unlikely to be functional.

If *piggyBac* movement among *T. ni*, *B. dorsalis*, and *S. frugiperda* occurred horizontally, then a straightforward analysis would suggest that the element occurred most recently in the species where conserved functional elements exist, which would be the cabbage looper moth. Thus far, only identical functional *piggyBac* elements have been discovered in this species. Conversely, the earliest appearance of *piggyBac* is suggested by the most degenerate elements which is inferred for *S. frugiperda* where none of the fragment sizes are consistent with a functional element. Thus, while *piggyBac* may have first appeared in the Lepidoptera, it most likely moved into the Diptera before entering the moth species *T. ni*. How this horizontal transmission may have occurred is quite intriguing, especially considering that these moth species and bactrocerid flies generally have distinct habitats (Lindgren et al., 1979; White and Elson-Harris, 1992). Given that *piggyBac* was first discovered by its ability to transpose from the *T. ni* genome into an infecting baculovirus, transmission via a viral intermediate must be given significant consideration. This type of transposition into baculoviruses is not unique, and has been observed for several other TTAA elements from *T. ni* and *S. frugiperda* cell lines (Beames and Summers, 1990; Fraser et al., 1983). Transposition of a Tc1-like transposon, TCp3.2, has also been observed in vivo from the *Cydia pomonella* larval host genome into an infecting granulovirus (Jehle et al., 1998). Notably, while the *T. ni piggyBac* was shown to be an autonomous functional element, the other “captured” transposons are thought to be defective and their transposition had to be catalyzed by a mobilizing (or cross-mobilizing) functional system. For inter-species transposon movement via a viral intermediate, transposition from the virus into the insect genome would also be required, and this has not been observed experimentally. While the host range of baculoviruses allows movement among lepidopterans, the question arises as to whether Lepidoptera and Diptera share common infectious agents. Presently, lepidopteran baculoviruses are known to have infection capability in dipterans (and other organisms), and in some cases abortive replication has been demonstrated (see Fraser, 2000). This may actually favor transmission of transposons if potential host species are able to survive the viral infection. Thus, a critical question relevant to interspecies transposon movement is whether a transposon within a baculovirus can transpose into a host genome during infection, and *piggyBac* within the AcNPV genome may provide a highly useful experimental system to test this possibility.

6.3. Implications for horizontal transmission of piggyBac

Beyond elucidating how *piggyBac* may have become distributed among insects, knowledge of how transposons move horizontally also has very important implications for the practical use of transposon vectors. An initial consideration is that any transgene instability that might precede interspecies movement would certainly affect the stability and function of transgenic strains cre-

ated for biological control. At worst, loss of the transgene would make such strains ineffective, and possibly exacerbate the targeted problem with their mass release (e.g. sterile males regaining fertility). Alternatively, transgene movement within the genome may place it under different position effects, altering expression of the vectored genes and thus changing the expected behavior of the strain. Of greater ecological concern is the potential for interspecies transgene movement, and thus one of the highest priorities for risk assessment of released transgenic insects is a determination of this potential. Transgene movement into non-target hosts may result in serious consequences to that species in terms of genomic stability and expression of vectored genes. Transposons are mutagenic agents, and if vectored into a host species having a mobilizing system, negative genomic affects may result (though one might argue whether this would be more deleterious than the mobilizing system). The unintended introduction of a genetic system into a non-target host must be evaluated on a case by case basis in terms of the genes involved and their activity in potential hosts, though transgene systems intended for destruction of pest species certainly would not be welcome in beneficial species. Conversely, transgenes intended to improve the health or reproduction of beneficial insects would be unwelcome in most pest species.

While we may be able to determine whether *piggyBac* or related cross-mobilizing systems exist in potential hosts and a range of other species, obviously this is not possible for all insects or other organisms. Nor can we exhaustively assess the potential for inter-species transfer mediated by viruses, bacteria, or other infectious agents. Since it is known that *piggyBac* can transpose from a eucaryotic to an infecting viral genome, it becomes the primary system in which to test the potential and frequency of this movement by non-autonomous elements having transposase provided from various sources. Nevertheless, for programs that envision the field release of *piggyBac* transformants in the near future, the most straightforward strategy to mitigate concerns for transgene stability and interspecies movement is to disable the vector's mobility properties post-integration.

7. Summary

In summary, *piggyBac* has been used to transform the germ-line of more than a dozen species spanning three orders of insects based on published and unpublished reports. Transformation frequencies have ranged from approximately 1–2% to greater than 40% per fertile G_0, though the lower frequencies will probably increase as investigators gain experience in methodology. Used in conjunction with one of several fluorescent protein markers, *piggyBac* vectors are likely to be universally applicable for gene transfer in insects. The precise nature of *piggyBac* transpositions, and the ability to remobilize genomic integrations supports the likelihood that *piggyBac* will be highly effective for genetic analysis, and in particular, insertional mutagenesis projects that will help identify, isolate, and analyze both genomic coding regions and regulatory elements. The *piggyBac* vector should also allow the creation of transgenic strains for use in biological control programs, though its existence in distantly related insect species raises concern for interspecies transfer of transgenes by horizontal transmission. Constructive assessment and mitigation of these concerns, as well as the most effective use of *piggyBac* for basic and applied studies will require continued analysis of *piggyBac* distribution, potential methods of interspecies movement, and the mechanisms involved in its transposition.

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