

Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector

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Abstract

Germline transformation of *Drosophila melanogaster* was attempted with the *piggyBac* gene-transfer system from the cabbage looper moth, *Trichoplusia ni*. Using a self-regulated transposase helper and a *white* marked vector, a transformation frequency of 1–3% per fertile G0 was obtained, similar to that previously achieved in the medfly. Use of an *hsp70*-regulated helper increased this frequency more than eight-fold. Transformation with a vector marked with *white* and green fluorescent protein (GFP) under polyubiquitin–nuclear localizing sequence regulation yielded seventy G1 transformants which all expressed GFP, but only twenty-seven of these expressed eye pigmentation that would have allowed their selection based on *white*⁺ expression. *PiggyBac* transformation in two distantly related dipteran species and efficient expression of the *gfp* marker supports the potential use of this system in other dipterans, and perhaps insects in general.

Keywords: *piggyBac* vector, germline transformation, green fluorescent protein, *Drosophila melanogaster*, position-effect variegation.

Introduction

The *piggyBac* transposable element from the cabbage looper moth, *Trichoplusia ni* (Cary *et al.*, 1989) has been shown to be an effective gene-transfer vector in the Mediterranean fruit fly, *Ceratitidis capitata* (Handler *et al.*, 1998). Use of an unmodified transposase helper under *piggyBac* promoter regulation in these experiments indicated that *piggyBac* retains autonomous function in the medfly, since transcriptional regulation was maintained, as well as enzymatic activity. This observation was novel since all

other successful insect germline transformations had been limited to dipteran species using vectors isolated from the same or another dipteran. The initial transformation of medfly (Loukeris *et al.*, 1995) used the *Minos* vector from *Drosophila hydei* (Franz & Savakis, 1991), and *Aedes aegypti* has been transformed with *Hermes* (Jasinskiene *et al.*, 1998) from *Musca domestica* (Warren *et al.*, 1994) and *mariner* (Coates *et al.*, 1998) from *Drosophila mauritiana* (Jacobson *et al.*, 1986). *Drosophila melanogaster* has been transformed as well by *Hermes* (O'Brochta *et al.*, 1996), *mariner* (Lidholm *et al.*, 1993), *Minos* (Franz *et al.*, 1994) and by the *P* and *hobo* transposons originally discovered in its own genome (see Berg & Howe, 1989). *Drosophila virilis* also has been transformed by *hobo* (Lozovskaya *et al.*, 1995; Gomez & Handler, 1997) and *mariner* (Lohe *et al.*, 1996). While the restriction to dipteran vectors is due in part to the limited number of transposon systems available from non-dipteran species, phylogenetic limitations on transposon function is not unexpected considering the deleterious effects functional transposons may have on a host genome. This is, indeed, reflected by the high level of regulation placed on transposon movement among species, among strains within a host species, and even among cell types within an organism (see Berg & Howe, 1989).

It is therefore worthwhile to consider whether *piggyBac* function in medfly is unique, or whether it also retains vector function in other dipterans. This is important to further understanding the phylogenetic regulation of *piggyBac* mobility, and the potential use of *piggyBac* vectors for gene transfer in other species. To test this possibility we attempted *piggyBac*-mediated germline transformation in *D. melanogaster*, which is distantly related to the medfly having diverged approximately 140 million years ago (Beverley & Wilson, 1984). Autonomous function was tested as it was in *C. capitata*, using a helper transposase under *piggyBac* promoter regulation.

The ability of *piggyBac* to function in several dipteran species will be supportive of its use in a wider range of insects, if not other organisms. However, the routine use of *piggyBac* will require optimization of vector function and transformant selection. Most other vector systems function optimally, or have been only tested with their helper transposase under *hsp70* promoter regulation. The

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transposition efficiency of most vectors has also been found to be influenced by the amount of internal DNA inserted, the position of this DNA within the vector, and the amount of subterminal DNA remaining in the vector. We have begun to test these variables, and in doing so optimizing the vector system by testing a transposase helper regulated by a heat-shock promoter, and varying the size and construction of *piggyBac* vectors.

Finally, the widespread use of *piggyBac* will be limited by the availability of easily detectable and unambiguous transformant markers. Most *Drosophila* transformations, as well as the few non-drosophilid transformations reported have depended on transformant selection by rescue of a mutant visible phenotype, usually eye pigmentation (see Ashburner *et al.*, 1998). Unfortunately, most insect species have neither visible mutant strains, nor the cloned DNA for the wild-type allele of the mutation, and these species require use of new dominant-acting marker genes which confer, preferably, a visible phenotype. Several markers of this type are available, although one which is most easily detected in living tissue and known to function well in heterologous systems is the gene for green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria* (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). Gene expression of most selectable markers has been typically enhanced by linkage to conditional *heat-shock protein* (*hsp*) promoters from *D. melanogaster* (Lis *et al.*, 1983), and while they are generally effective in non-drosophilid species, new promoters will simplify rearing and enhance strategies for transformant selection and creation of new phenotypes. To begin to address these questions, we have tested a modified transformation marker within *piggyBac*, linking an enhanced *gfp* (Cormack *et al.*, 1996; Yang *et al.*, 1996) to the polyubiquitin promoter from *D. melanogaster* (Lee *et al.*, 1988) and the nuclear localizing sequence signal peptide from SV40 (Lanford *et al.*, 1986). To test the relative influence of position-effect variegation on this marker compared to more typical visible eye-colour markers, we have compared the transgenic expression of GFP to *white*⁺.

Results

Transformation experiments

In the first of three transformation experiments we tested the *piggyBac* vector system in a *D. melanogaster white*

strain using a helper transposase under *piggyBac* regulation (pBΔSac) and a vector marked solely with a *D. melanogaster* mini-*white* gene (pB[Dmw]). A mixture of vector and helper plasmids at concentrations of 600 and 400 µg/ml, respectively, was injected into 2650 embryos from which 418 larvae hatched with 283 emerging as adults (Table 1). The G0 adults were backcrossed to *w[m]* flies in groups, totalling 111. Four of the G0 lines yielded G1 offspring having varying levels of eye pigmentation (Fig. 1). One line (F30) was sterile, and one line produced only white-eye offspring, and therefore only two of the putative *Dm[pBw]* transformants were verified. One of these (F13) exhibited eye pigmentation only in females in several succeeding generations, suggesting that the integration caused a sex-linked lethal mutation. Presuming a fertility rate of 50% (fertility rates are typically between 40 and 60%; see below), an approximate transformation frequency of 1–3% of fertile G0s was obtained.

In the second experiment, the pB[Dmw] vector was again tested, but with a *piggyBac* transposase helper under *D. melanogaster hsp70* (Lis *et al.*, 1983) promoter regulation (phsp-pBac). A vector/helper mixture, at a concentration of 600/400 µg/ml, was injected into 1940 embryos, of which 247 larvae hatched, with 122 emerging as adults (Table 1). G0 adults were initially backcrossed in a total of forty-nine groups to *w[m]* flies, after which they were individually mated to determine fertility. Of ninety-eight surviving G0 flies, forty-one yielded offspring resulting in a fertility rate 42%. Of the forty-one fertile G0 flies, eleven lines produced offspring having varying levels of eye coloration (Fig. 1) yielding a transformation frequency of 26%. The number of G1 offspring from the G0 lines varied considerably, ranging from one G1 in lines M11 and F1, to 102 G1 flies in line M13.

In the third experiment the phsp-pBac helper was used, but with a *piggyBac* vector including the enhanced *green fluorescent protein* (*gfp*) marker gene in addition to the *D. melanogaster white* gene. This allowed the testing of a new *gfp* marker construct in transformants that could be primarily identified by *white* expression. Although expression of wild-type GFP under polyubiquitin–nuclear localizing sequence regulation had been tested previously in *D. melanogaster P* transformants (Davis *et al.*, 1995), we sought to improve expression by use of an enhanced GFP (EGFP-1) having a double mutation causing a reported

Table 1. Transformation experiments.

Expt.	Vector/helper	Eggs injected	G0s mated	% fertility	No. G0 lines	No. G1	Transformant. lines frequency
I	pB[Dmw]/pBΔSac	2650	283	nd	4	11	0.01–0.03*
II	pB[Dmw]/phsp-pBac	1940	122	42	11	266	0.26
III	pB[Dmw, PUbnlEGFP]/phsp-pBac	2147	218	nd	7	70	0.06–0.07*

*Estimated frequency based on 50% fertility.

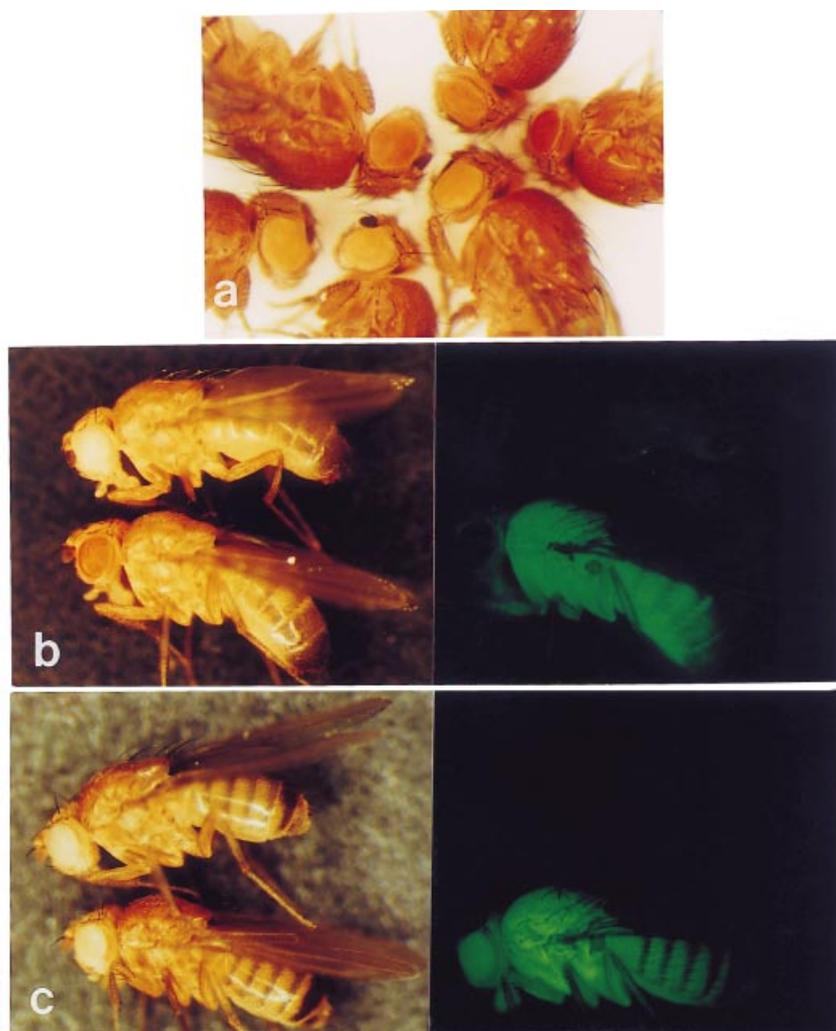


Figure 1. (a) Eye-colour phenotypes of *Dm[pBw]* transformants (b) a *w[m]* host strain fly (top) and orange-eye *Dm[pBw, gfp]* transformant fly (bottom) under brightfield (left) and UV light (right), and (c) a *w[m]* host strain fly (top) and white-eye *Dm[pBw, gfp]* transformant fly (bottom) under brightfield (left) and UV light (right).

increase in expression of up to thirty-fivefold (Cormack *et al.*, 1996; Yang *et al.*, 1996). The variant form is also optimized for mammalian codon usage and polyadenylation, and preliminary tests of the marker construct indicated transient GFP expression in both *Drosophila* embryos and dipteran and lepidopteran cell lines (A. M. Handler and R. A. Harrell, unpublished). The vector construct, pB[Dmw, PUBnlsEGFP], also allowed evaluation of *piggyBac* transformation with a 10.0-kb vector, approximately 3.4 kb larger than previous vectors tested, and having 748 bp of *piggyBac* DNA deleted (previous vectors retained all *piggyBac* DNA). As before, a mixture of 600 $\mu\text{g/ml}$ vector and 400 $\mu\text{g/ml}$ helper was injected into 2147 embryos, of which 412 larvae hatched and 218 emerged as adults (Table 1). G0 adults were backcrossed to *w[m]* flies in a total of ninety mating groups, of which seventy-nine yielded offspring. Although we depended upon *white* expression as the primary marker, G1 larvae and pupae were examined under UV for visible GFP expression, and

Table 2. G1 *white*⁺ and GFP marker expression in *Dm[pBw, gfp]* transformants.

G0 line	No. G1	GFP	<i>white</i> ⁺	Frequency <i>white</i> ⁺
M4	4	4	3	0.75
M9	21	21	2	0.10
M12	3	3	1	0.33
M23	15	15	14	0.93
M45	5	5	0	0
M47	21	21	6	0.29
F10	1	1	1	1.00
Total	70	70	27	0.39

seven of the G0 lines yielded fluorescent G1 larvae and pupae. Interestingly, as shown in Table 2, upon adult emergence only six of the seven G0 lines yielded G1 offspring with observable *white*⁺ eye colour pigmentation. While seventy G1 offspring in total exhibited observable

green fluorescence, only twenty-seven of these flies exhibited a level of eye pigmentation that would have allowed their selection under normal screening procedures. In contrast, all of the *white*⁺ G1 flies expressed GFP. Figure 1(b) shows a *Dm*[*pBw*, *egfp*] transformant having an orange eye colour and GFP fluorescence, with no fluorescence observed in the *w*[*m*] host. Figure 1(c) shows another transformant having a white-eye phenotype indistinguishable from that in the *w*[*m*] host strain, but exhibiting an equal, if not greater level of GFP fluorescence compared with the orange-eye transformant. Notably, fluorescence is quenched in the eye of the pigmented transformant, while it is easily visible in the white-eye transformant. High-magnification examination revealed a few pigmented ommatidia in some white-eye G1 flies expressing GFP, although these would not have been normally detected. Based on selection by GFP expression and presuming 50% fertility, an approximate transformation frequency of 6–7% of fertile G0 flies is deduced.

Southern analysis

Genomic transposition of the *piggyBac* vectors was verified by Southern DNA hybridization. The basic strategy was to perform hybridizations to the 5' vector arm using the *piggyBac* *Sph*I-*Hpa*I or *Nsi*I-*Hpa*I fragment as probe, and the 3' vector arm using the *Hpa*I-*Ase*I or *Hpa*I-*Nsi*I fragment as probe. Using probes to both vector arms, internal fragments spanning most of the vector were detected. Hybridizations to the vector arms and adjacent chromosomal sequence indicate their presence in non-plasmid DNA and indicate the number of integrations, while internal hybridizations that yield known fragment sizes confirm vector integrity.

pB[*Dmw*]. For the *pB*[*Dmw*] transformants, genomic DNA was initially digested with *Bgl*II and hybridized to the labelled *Sph*-*Hpa* *piggyBac* fragment, which detects both vector arms resulting in two bands for each integration (Fig. 2A). Each intact vector integration should result in one band greater than 0.67 kb for the 5' arm, and one band greater than 5.9 kb for the 3' arm. Because we observed varying eye-colour phenotypes among G1 sublines, and in some cases within G1 sublines, sublines having light-orange, dark-orange or red eye coloration from the same G1 sublines were selected for hybridization analysis. For example, flies having differing phenotypes from lines M13–39, M19–90 and M19–91 were hybridized separately, but no difference in the number or sites of insertion were apparent. Of all the lines tested, all had single integrations except for two lines having two integrations (M13–39 and M19–91) and one line having three integrations (F14–63). All the lines with multiple integrations had dark-orange or red eye colour, although several lines with a single integration also shared these phenotypes. Hybridization patterns for the lines tested indicated that for most

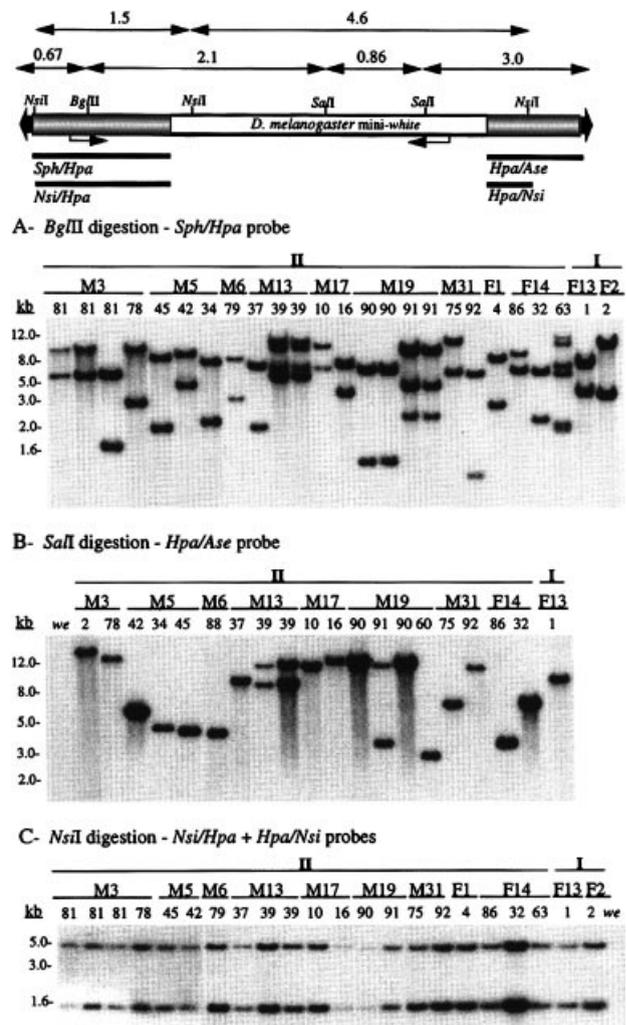


Figure 2. Southern DNA hybridization analysis of *Dm*[*pBw*] transformant sublines, and *w*[*m*] host strain control samples from transformations using the *pB*Δ*Sac* (experiment I) or *phsp*-*pBac* (experiment II) helpers. At the top is a schematic (not to scale) of the *pB*[*Dmw*] vector showing the *Bgl*II, *Sal*I, and *Nsi*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars; see Experimental procedures). Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded grey, and the *mini-white* marker gene is white. DNA size markers are shown to the left of the autoradiograms. M and F designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

of the G0 lines, different integrations were transmitted to many of the G1 sibling offspring. For example, the three G1 sublines tested from both the M3 and M5 G0 lines all show different patterns indicating at least three independent integrations occurring in the two G0 germlines.

Genomic DNA digested with *Sal*I and hybridized to *Hpa*-*Ase*I probe yielded single bands greater than 3.0 kb for each integration, and the number of integrations determined were consistent with the *Sph*-*Hpa*I hybridizations (Fig. 2B). For all samples, *Nsi*I digestion and hybridization

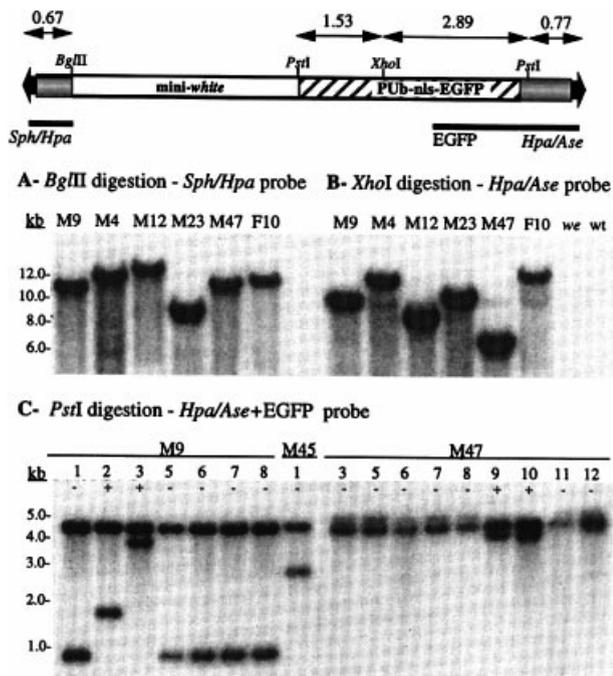


Figure 3. Southern DNA hybridization analysis of *Dm*[*pBw*, *gfp*] transformant sublines, and wild-type (wt) and *w*[*m*] host strain control samples. At the top is a schematic (not to scale) of the pB[Dmw, PUBnlsEGFP] vector showing the *Bgl*III, *Xho*I and *Pst*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars; see Experimental procedures). The *Sph*/Hpa probe contains 0.67 kb of vector sequence (*Sph*I to *Bgl*III) with *Bgl*III to *Hpa*I *piggyBac* sequence deleted from the vector. Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded grey, the *mini-white* marker gene is white, and the EGFP marker gene is hatched. DNA size markers are shown to the left of the autoradiograms. M and F designations refer to G0 lines with selected G1 transformant progeny for samples in blots A and B. For blot C, specific G1 line numbers are given below, with the designation (+) for those expressing visible eye pigmentation and (-) for those having non-pigmented white eyes.

to *Nsi*I-*Hpa*I and *Hpa*I-*Nsi*I probe yielded only 1.5 kb and 4.6 kb bands accounting for 6.1 kb of the 6.6-kb vector, indicating the same generally high level of vector integrity for all integrations tested.

pB[*Dmw*, *PUBnlsEGFP*]. G1 sublines from six G0 lines transformed with the pB[Dmw, PUBnlsEGFP] vector were digested with either *Bgl*III and probed with *Sph*I-*Hpa*I for 5' vector arm analysis, or digested with *Xho*I and probed with *Hpa*I-*Ase*I for 3' arm analysis (Fig. 3A, B). Both hybridizations yielded one band for each sample, indicating single integrations having occurred in each line. *Nsi*I restriction digests with *Nsi*I-*Hpa*I and *Hpa*I-*Nsi*I hybridizations yielded 0.7 kb and 0.8 kb bands indicating vector integrity for each integration (data not shown).

Two G0 lines, M9 and M47, yielded a high proportion of G1 flies expressing only GFP and white eyes, and line M45 which yielded only white-eye transformants. These

lines were analysed by *Pst*I digestion and hybridization to EGFP and *Hpa*-*Ase*. All lines shared the 4.4 kb internal vector fragment, with an additional junction fragment from the 3' vector arm and adjacent insertion site chromosomal DNA. The M9 white eye lines all shared the same integration indicated by a 0.9-kb junction fragment, and similarly the M47 white eye lines all shared the same 5.0-kb junction fragment. The pigmented lines M9-2 and M9-3 had different integrations from each other, and from their white-eye sibling lines, and the pigmented lines M47-9 and M47-10 shared the same integration based on a 4.0-kb junction fragment, but which differs from their white-eye siblings. These hybridizations, and that for M45-1, proves that the white-eye flies were transformed, and that *white* expression was likely influenced by differing insertion sites from their pigmented sibling lines.

Insertion-site sequences

To verify that *piggyBac*-mediated chromosomal transpositions had occurred, insertion sites were isolated by inverse polymerase chain reaction (PCR) from sublines F1-2, M17-4 and M31-6, all having single integrations. Subcloned PCR products were sequenced and compared to *piggyBac* terminal sequences by DNA alignment and BLAST analysis (Altschul *et al.*, 1990) to identify genomic insertion site sequences and distinguish them from those in the injected plasmids. For all the integrations both the 5' and 3' junctions yielded the *piggyBac* inverted terminal repeat sequences immediately adjacent to a TTAA sequence and proximal insertion site DNA (Fig. 4). The TTAA duplicated target site is characteristic of all *piggyBac* integrations (Elick *et al.*, 1995), and typically indicates a vector-mediated transposition. The BLAST analysis revealed that the M17-4 integration occurred in a TTAA site within the *cubitus interruptus*-Dominant gene located on chromosome 4 at nucleotide 12 898 (GenBank submission U66884; Ahmed & Podemski, 1977), and the M31-6 integration was found to have occurred in a TTAA site within a previously sequenced region of the distal X chromosome (GenBank submission AL009193; L. Murphy, D. Harris & B. Barrell, direct submission). Determination of insertions in these previously sequenced sites gives the first direct proof that a *piggyBac* vector does indeed insert into and duplicates TTAA insertion sites in a eucaryotic genome.

Discussion

We show here that the *piggyBac* transposable element from the cabbage looper moth can act as an effective gene-transfer vector system in *D. melanogaster*, functioning at a frequency equivalent to that in another dipteran species, *Ceratitidis capitata* (Handler *et al.*, 1998). Notably, autonomous *piggyBac* function, demonstrated by use of a self-regulated *piggyBac* transposase helper, was shown

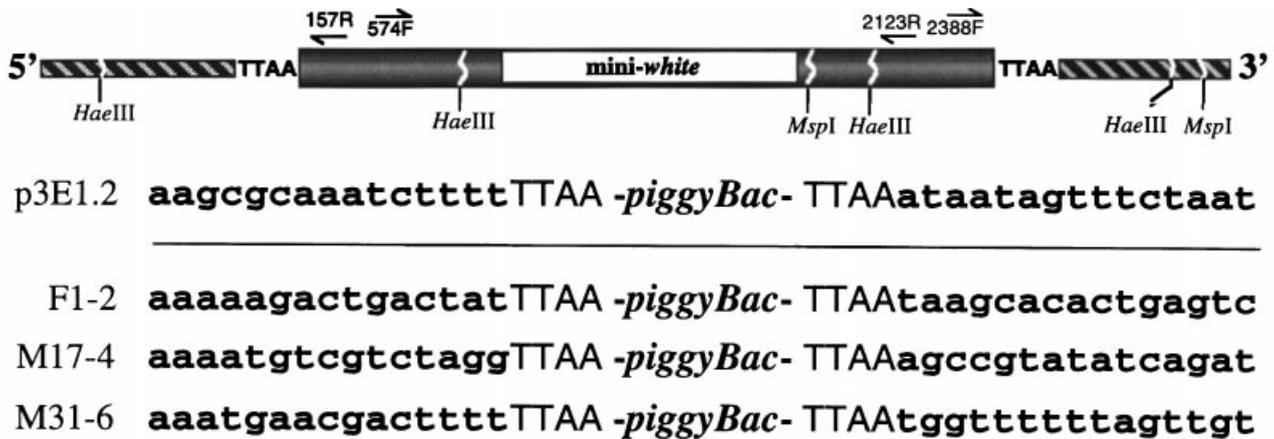


Figure 4. Inverse PCR strategy to isolate and sequence the pB[Dmw] vector insertion site in transformant sublines. At the top is a schematic (not to scale) of the vector insertion in the host plasmid showing the approximate location of the restriction sites and primers used for PCR. Forward (F) and reverse (R) primers are numbered according to their nucleotide position in *piggyBac*. The *piggyBac* sequence is shown in grey surrounded by the TAA duplicated insertion site, the mini-*white* marker gene is white, and chromosomal sequence is hatched. Below is the *piggyBac* insertion site sequence in p3E1.2, and the proximal insertion site sequences for three of the transformant sublines.

in both of these distantly related species suggesting the likelihood that *piggyBac* function is widespread among dipterans, if not other insect orders. Use of the non-modified helper in the two species has resulted in consistent, albeit relatively low transformation frequencies of less than 5%. The heat-shock regulated helper, however, increased the transformation frequency by eightfold in *Drosophila*, indicating that the *piggyBac* system could be as effective as routinely used systems such as *P* and *hobo* which have been thus far inactive in non-drosophilids (see O'Brochta & Atkinson, 1996). Increasing vector size by one-third, for at least one experiment, served to decrease *piggyBac* transformation frequency by two-thirds, and thus the general influence of conditional helper expression and vector size on *piggyBac* transposition is consistent with other systems. Further analysis will define the limits for vector size and requisite *piggyBac* sequences necessary for efficient transposition. While vector function should be improved, these results remain very encouraging for the routine use of *piggyBac*-mediated gene transfer in additional insect species.

For some G0 lines a large number of G1 transformant offspring were observed, and while most of them had single integrations, many of the integration sites in the sibling G1 transformants were different owing to independent transposition events, resulting also in varying eye-colour phenotypes. This differs from the medfly transformations where multiple G1 lines occurred, but they typically shared the same integration, with some having an additional second integration. Irrespective of the number of integrations, the medfly eye-colour phenotypes were generally consistent among G1 siblings. A straightforward clonal analysis of these differing results suggests that fewer integrations occurred in medfly, but they occurred

early in gametogenesis resulting in multiple G1 flies sharing common integrations. In *Drosophila*, more integrations occurred, but probably occurred late in gametogenesis. More transformation experiments will determine if there are true species-specific (or genome-specific) differences in the timing and frequency of *piggyBac* transposition, or whether this reflects procedural differences in transformation of the two species.

An assessment of vector activity based on germline transformation frequency is a factor of both transposon mobility in the host embryo and levels of genomic position-effect suppression of the marker gene, or stated more simply, the ability to visibly identify putative transformants. While position-effect variegation and suppression of *white* expression in transformants is well established (Hazelrigg *et al.*, 1984; Pirrotta *et al.*, 1985), the effect of complete marker suppression on transformation frequencies has not been assessed since such transformants have been only detected fortuitously after molecular analysis. The experiment using both the *white* and GFP markers proved the importance of position effects on marker expression convincingly, since GFP was readily detected in seventy G1 flies, yet eye pigmentation was apparent in less than 40% of these. Under typical screening procedures these flies would not have been scored as transformants, though pigmentation in a few ommatidia in some flies could be detected at high magnification, and for a few lines pigmentation was more apparent in subsequent generations. It is likely that expression of the *white* marker would have been improved by heat-shock regulation, but nonetheless GFP was easily detectable in all the non-pigmented transformants, and strongly expressed in some. The influence of modifier genes on position-effect variegation is complex, and target genes (or their promoters) are not equivalently

affected (Bhadra *et al.*, 1998). The polyubiquitin-*gfp* gene may be a target of position effect modifiers, but it is clearly less susceptible to suppression relative to *white* in terms of its expressed phenotype in the same chromosomal context. How this relates to actual gene expression and whether it relates to the *piggyBac* vector and its integration specificities is unknown at this time. Regardless, the data suggest that GFP is a more reliable visible marker than *white*, which portends well for its use as a general marker in other insect systems.

While GFP expressivity is critical for non-drosophilid species not amenable to mutant rescue, it also widens the possibility for using the dominant expression of GFP as a primary transformant marker in many *Drosophila* lines not already carrying the *white* or *rosy* mutations, or for screens requiring selection in early development. Although vectors carrying *white* and *gfp* have been tested previously, the transformations that we are aware of used only *white* as the transformant selection, with GFP assessed secondarily for specific spatial or developmental expression (Wang & Hazelrigg, 1994; Davis *et al.*, 1995). Thus, inconsistent marker expression in these strains was probably not apparent. Use of GFP as an efficient primary marker requires a dedicated optical system that may remain a barrier to its general use, but nevertheless, it should certainly find great usefulness in transforming non-mutant lines, previously transformed lines, and with inefficient vectors that would benefit from sensitive selections.

Two of the three insertion sites that were sequenced were found to be in previously sequenced genomic loci, and as expected the insertion sites were all TTAA with one of them within the *ci^D* allele on the fourth chromosome. Many transposons have insertion site preferences, and for at least some, a clear negative bias against specific sites or loci. This has been clearly demonstrated by genomic hotspots and coldspots for *P* integration in *D. melanogaster* (see Engels, 1989), and by differences in preferential integration sites between *hobo* and *P* (Smith *et al.*, 1993). If the TTAA specificity for *piggyBac* integration is not further influenced by proximal sequences, then *piggyBac* transpositions may find use in transposon mutagenesis and enhancer traps for loci refractory to *P* or *hobo* transpositions in *Drosophila*. A similar application for genetic analysis in non-drosophilid species is now possible, and is certainly a high priority.

Experimental procedures

Insect strains and rearing

The *D. melanogaster white* strain *w[m]* and transformant progeny were maintained at 23–25 °C on standard cornmeal–yeast–molasses media.

Plasmids

The *piggyBac* transposase helper plasmid, p Δ Sac, having its 5' terminus deleted was described previously (Handler *et al.*, 1998). A transposase helper under heat-shock promoter regulation was created by isolation of the 457-bp *XbaI-XmnI* 5' non-translated sequence from the *hsp70* gene (Lis *et al.*, 1983). The *XbaI-XmnI* fragment was blunted and ligated into the *SacI*-blunted site of p Δ Sac to create phsp-pBac. This places the *hsp70* promoter sequence upstream of the putative *piggyBac* promoter.

The pB[Dmw] vector was created by insertion of a *D. melanogaster* mini-*white* gene (Pirrotta *et al.*, 1985) into the 3E1 *piggyBac* element within the 6.0-kb p3E1.2 plasmid (Cary *et al.*, 1989). The mini-*white* gene was isolated as a 4.2-kb *EcoRI* fragment, blunted and ligated into the p3E1.2 *HpaI* site. The inserted *w* gene interrupts the *piggyBac* open reading frame, but otherwise leaves the *piggyBac* element intact, with the respective promoters in opposite orientation. A *piggyBac* vector marked with *w* and *gfp* was created by initial construction of *piggyBac* marked with an enhanced *gfp* regulated by the *D. melanogaster* polyubiquitin (PUB) promoter (Lee *et al.*, 1988) linked in-frame to the SV40 nuclear localizing sequence (nls) (Lanford *et al.*, 1986). The polyubiquitin-nls (PUB-nls) cassette from PUBnlsGFP (Davis *et al.*, 1995) was isolated as *KpnI-SmaI* fragment and inserted into the *KpnI-SmaI* cloning site of EGFP-1 (Clontech) (Cormack *et al.*, 1996; Yang *et al.*, 1996). Polyubiquitin-nls-EGFP was then isolated as a 4.1-kb *BglII-StuI* fragment and ligated into the *BglII-HpaI* site of *piggyBac* within p3E1.2 to create pB[PUBnlsEGFP]. The mini-*white* gene was then inserted into the unique *BglII* site by blunt-end cloning to create pB[Dmw, PUBnlsEGFP].

Injections

Embryo injections used standard procedures (Rubin & Spradling, 1982) with dechorionation achieved either manually or by 1.6% hypochlorite solution followed by several washes in 0.02% Triton-X 100. Eggs were placed on double-stick tape, desiccated in room air and injected under Halocarbon 700 oil. DNA mixtures had vector/helper concentrations of 600 : 400 μ g/ml in injection buffer (5 mM KCl; 0.1 mM sodium phosphate pH 6.8). Injected eggs were placed in an oxygenated and humidified tissue culture chamber at 22–23 °C, and phsp-pBac injected eggs were heat shocked at 37 °C for 1 h at 3–6 h after injection. Eclosed G0 adults were mated either individually to two or three *w[m]* adults, or in groups of three females to six males. G1 eggs were collected for 2 weeks and reared under standard conditions.

Southern hybridization

Five to ten micrograms of genomic DNA were digested with indicated restriction enzymes and separated on 0.8% agarose gels. DNA was stained with ethidium bromide, blotted to nylon filters and immobilized by UV irradiation. Hybridization probes were labelled with ³²P-dCTP by random priming (Gibco BRL) according to the manufacturer's specifications. Probe DNA was generated from indicated *piggyBac* restriction fragments that were separated from p3E1.2, or the entire *egfp* gene from pEGFP-1 (Clontech), by agarose electrophoresis and gel elution. Hybridizations were performed in phosphate buffer pH 7.5; 1% bovine serum albumin (BSA); 7% sodium dodecyl sulphate (SDS) at 65 °C with an initial wash in 2 \times SSC; 0.2% SDS at room temperature and two washes in 1 \times SSC; 0.1% SDS at 55 °C for 30 min.

Autoradiography was performed by exposure on Kodak X-Omat film at -90°C .

Polymerase chain reaction and sequence analysis

Inverse PCR was performed as described previously (Handler *et al.*, 1998) using *Hae*III digestions for 5' and 3' junctions and *Msp*I digestion for 3' junctions. Subcloned PCR products were sequenced and analysed by alignment using GeneWorks 2.5 software (Oxford Molecular Group) and subjected to BLAST analysis (Altschul *et al.*, 1990).

Green fluorescent protein analysis

GFP was observed at all developmental stages under a Leica MZ-12 stereozoom microscope using a mercury lamp and a UV light longpass filter set (HQ 41012 FITC; Chroma) optimized for red-shifted GFP variants. Photographic documentation used an Olympus OM-4 camera and 400 ASA Fujichrome film with exposure times that were determined empirically.

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