

Tn5 as an insect gene vector

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Abstract

The purpose of this study was to explore alternatives to insect-derived transposable elements as insect gene vectors with the intention of improving existing insect transgenesis methods. The mobility properties of the bacterial transposon, *Tn5*, were tested in mosquitoes using a transient transposable element mobility assay and by attempting to create transgenic insects. *Tn5* synaptic complexes were assembled in vitro in the absence of Mg^{2+} and co-injected with a target plasmid into developing yellow fever mosquito, *Aedes aegypti*, embryos. Target plasmids recovered from embryos a day later were screened for the presence of *Tn5*. Recombinants (transposition events) were found at a frequency of 1.2×10^{-3} . Some transposition events did not appear to be associated with canonical 9 bp direct duplications at the site of insertion and also were associated with either deletions or rearrangements. A *Tn5* element containing the brain-specific transgene, *3xP3DsRed*, was assembled into synaptic complexes in vitro and injected into pre-blastoderm embryos of *Ae. aegypti*. Of the approximately 900 embryos surviving injection and developing into adults, two produced transgenic progeny. Both transgenic events involved the co-integrations of approximately five elements resulting in nested and tandem arrayed *Tn5::3xP3DsRed* elements. This study extends the known host range of *Tn5* to insects and makes available to insect biologists and others another eukaryotic genome-manipulation tool. The hyperactivity of synaptic complexes may be responsible for the unusual clustering of elements and managing this aspect of the element's behavior will be important in future applications of this technology to insects.

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1. Introduction

Germ-line transformation systems are currently available that permit the introduction of exogenous DNA into insect genomes and allowing its stable, vertical transmission from generation to generation. The systems are all based on Class II transposable elements (Finnegan, 1989) isolated originally from insects. The efficiency of these systems and the ease with which one can produce transgenic insects varies (Atkinson et al., 2001). Germ-line transformation of *Drosophila melanogaster* using the *P* or *Hermes* elements can be very efficient, with transformation frequencies of 50% and higher being reported (O'Brochta et al., 1996). Trans-

formation frequency is taken as the percentage of adults developing from injected embryos (G_0 s) that produce transgenic progeny. The performances of *mariner* and *piggyBac* in *D. melanogaster*, however, are less impressive with transformation frequencies of 10% or less being reported (Handler and Harrell, 1999; Li et al., 2001). The performances of these transformation systems in insects other than *Drosophila* are also variable. For example, *piggyBac* transforms *An. gambiae* with a frequency of about 1% (Grossman et al., 2001), *Ae. aegypti* at approximately 4% (Lobo et al., 2002), *An. stephensi* at approximately 10–20% (O'Brochta, unpublished data) and *An. albimanus* at approximately 50% (Perera et al., 2002).

Efforts to increase the rates of both transpositional recombination and genetic transformation in individual species have had limited success. For example, mutations in the transposase of the *Himar* element, a

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mariner-like element from the fly *Haematobia irritans*, have been found to increase the rates of transposition of this element in *E. coli* but not in insects (Lampe et al., 2000). Creating *mariner* elements in which the 5' inverted terminal repeat (ITR) is replaced with a 3' ITR with a higher binding affinity for *Mos1* transposase results in an increase in the transposition activity of the element in *E. coli* (Auge-Gouillou et al., 2001). Kapetanaki et al. (2002) report higher rates of *Minos*-mediated transformation of *D. melanogaster* and *Ceratitidis capitata* following the co-injection of *Minos* transposase mRNA and a *Minos* vector compared to when a *Minos*-containing helper plasmid was used. These data are encouraging in that they illustrate the potential for existing insect vector systems to be improved through modification. A deeper understanding of how existing transposable element-based vector systems function in foreign hosts and how such systems can be modified to be more effective is warranted and desirable. Furthermore, the continued testing of alternative recombination systems (such as Class II transposable elements from non-insect systems) as insect gene vectors will not only potentially increase the number of functional transformation vectors available, but may also lead to the discovery of systems that are naturally hyper-active in insects.

A number of transposable element systems of microbial origin appear to hold potential as insect gene vectors. Several systems, including those derived from *Ty1* (Devine and Boeke, 1994), *Tn5* (Goryshin and Reznikoff, 1998), *Tn7* (Gwinn et al., 1997) and *Mu* (Haapa et al., 1999) are commercially available and are used for a variety of genomics applications (Boeke, 2002). Here, we report the results of testing the *Tn5* system in an insect.

Tn5 is a prokaryotic composite transposon isolated originally from enteric bacteria and is 5.8 kb in length. The 1.5 kb of terminal sequences found at the right and left ends are IS50 (IS50L, IS50R) insertion sequences. IS50R is a fully functional insertion sequence that encodes a 476 amino acid transposase and contains 19 bp terminal inverted repeats (outside end, OE and inside end, IE). *Tn5* transposition occurs via a multi-step, cut-and-paste mechanism. The initial step involves binding of transposase to the terminal inverted repeats of the donor element, followed by synapsis of the terminal inverted repeats and transposase. The element is excised from the donor site and results in a synaptic complex consisting of the transposable element and a dimer of transposase (Fig. 1). In the presence of Mg^{2+} , the synaptic complex is an active intermediate which, upon association with target DNA, undergoes a series of strand transfer reactions with the target molecule, resulting in the integration of the element, the creation of a 9 bp direct duplication of the target site and release of transposase. Mutations in transposase, as

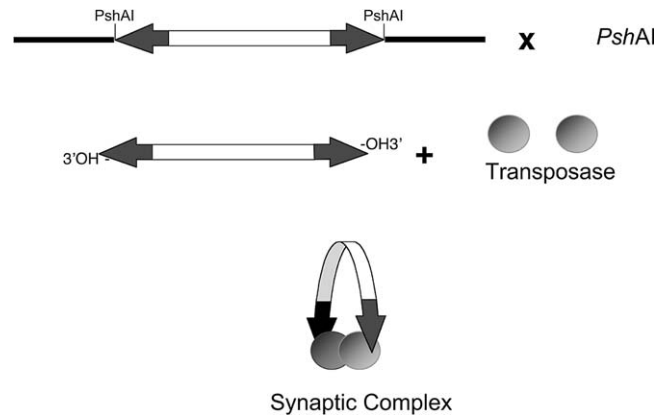


Fig. 1. Creation of synaptic complexes in vitro. A plasmid containing the *Tn5* vector is digested with the restriction endonuclease, *PshAI*, to yield a blunt-ended fragment including precisely the *Tn5* vector with 3' OH. The excised element is mixed with purified *Tn5* transposase in vitro resulting in an inactive synaptic complex that will become active (transpositionally competent) in the presence of Mg^{2+} .

well as sequence alterations in the OE and IE, have resulted in a transposon system that is 1000-fold more active in vitro than the unmodified *IS50* element (Goryshin and Reznikoff, 1998). Introduction of preformed transposon/transposase synaptic complexes into several species of Gram-negative bacteria and the yeast *Saccharomyces cerevisiae* by electroporation have resulted in the integration of *Tn5* into the genomic DNA of these hosts via the *Tn5* transposition reaction (Goryshin et al., 2000).

Current insect transformation protocols call for microinjecting a mixture of two plasmids into pre-blastoderm embryos to achieve transformation. One of these plasmids contains a non-autonomous transposable element composed of fully functional ITRs flanking the transgenes and genetic markers of interest, while the second plasmid contains the transposase gene under the regulatory control of a strong promoter. Transient expression of the transposase gene is required post-injection in order for element excision and integration to occur. Previous experiments examining the frequency of excision of elements such as *Hermes*, *mariner*, *Minos* and *piggyBac* from plasmids injected into insect embryos along with a "helper" plasmid indicate that only approximately one plasmid per 1000 injected will result in an excision event (Atkinson et al., 2001). Therefore, most of the donor molecules introduced into insect embryos during the transformation process will contribute nothing to the transformation efforts. The ability to introduce pre-excised elements configured as active intermediates, such as synaptic complexes, may result in higher integration rates, thereby improving the overall efficiency of transformation. The *Tn5* system provided an opportunity to examine the behavior of pre-assembled

synaptic complexes as the starting point in the process of creating transgenic insects and the effects on transformation rates.

2. Materials and methods

2.1. *Aedes embryonic mobility assays*

2.1.1. Injection cocktail

The injection cocktail consisted of a freshly prepared mixture of 1 μ l (1 unit) of EZ::TN[™]<KAN-1>Tnp Transposome[™] (Epicentre Technologies) and 4 μ l of a 0.625 μ g/ μ l solution of pUCSacRB (O'Brochta et al., 1994). EZ::TN[™]<KAN-1>Tnp Transposome[™] is a stable synaptic complex consisting of EZ::TN[™] Transposase and a pre-excised EZ::TN transposon containing a kanamycin-resistance gene (KanR). The transposome is at a concentration of 25 μ g/ μ l in 50% glycerol; 27.5 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.5 mM dithiothreitol; 0.3 mM EDTA; 0.05% Triton X-100. pUCSacRB contains the *Bacillus subtilis sacRB* gene in pUC19 (ampicillin resistance) and encodes for levansucrase (β -2,6-fructan: D-glucose-1-fructosyl-transferase; EC 2.4.10) which, when expressed in *Escherichia coli* in the presence of at least 5% sucrose, results in cell lysis (Gay et al., 1985; O'Brochta et al., 1994). pUCSacRB serves as a transposition target plasmid in these experiments (O'Brochta et al., 1994) and insertions into *SacRB* resulting in a loss of gene expression results in sucrose resistance. The details of this plasmid-based transposable element mobility assay have been described elsewhere (O'Brochta et al., 1994).

2.1.2. Injection and post-injection procedures

Pre-blastoderm embryos of the wild-type *Aedes aegypti* strain, *Orlando*, were injected with the injection cocktail essentially as described (Morris et al., 1989) and allowed to develop at 28 °C for approximately 24 h, at which time the embryos were collected and low molecular weight DNA was isolated by the Hirt method (Hirt, 1967). Recovered DNA was resuspended in water and used to transform *E. coli* (strain DH10B) by electroporation. Transformants were incubated in 1 ml of LB broth at 37 °C for 1 h at which time they were plated (100 μ l/plate) on LB containing ampicillin (0.1 mg/ml; selecting for target plasmids), kanamycin (0.05 mg/ml; selecting for EZ::TN[™]<KAN-1>) and sucrose (10%; selecting for mutations in *SacRB*). An aliquot (100 μ l) of the cells was also plated on LB containing ampicillin (0.1 mg/ml) to permit the number of target plasmids recovered from the injected embryos to be estimated. Ampicillin-kanamycin-sucrose-resistant colonies were scored as putative transpositional integration events and were further analyzed by restriction mapping and DNA sequencing.

2.2. *Aedes germ-line transformation*

2.2.1. Vector construction

The plasmid EZ::TN[™]pMOD[™]-2<MCS> (Epicentre Technologies) was modified by ligating a short oligonucleotide containing an *FseI* and *AscI* site between the *EcoRI* and *PstI* sites, resulting in the plasmid pTn5AscFse. The *3xP3DsRed* gene consisting of the brain- and ventral ganglion-specific promoter *3xP3* (Berghammer et al., 1999) and the gene for the auto-fluorescent protein *DsRed* (Clontech) was amplified from the plasmid pBac(3xP3-DsRed, UASp-EYFP-K10) (Horn et al., 2003) using primers containing an *AscI* restriction site and inserted into pCR[®] 2.1 (Invitrogen) to create the plasmid pTA::3xP3DsRed. *3xP3DsRed* was removed from pTA::3xP3DsRed as an *AscI* fragment and inserted into the *AscI* site of pTn5AscFse to create the plasmid pTn5::3xP3DsRed (Fig. 2). The constructed plasmids were confirmed by PCR amplification and restriction endonuclease mapping.

2.2.2. Injection cocktail

pTn5::3xP3DsRed was digested to completion with *PshAI*, resulting in a 1.4 kb blunt-ended fragment containing the *Tn5::3xP3DsRed* transposon. The pre-excised transposon was gel purified and mixed with EZ::TN[™]Transposase, a hyper-active form of *Tn5* transposase (Goryshin and Reznikoff, 1998), and

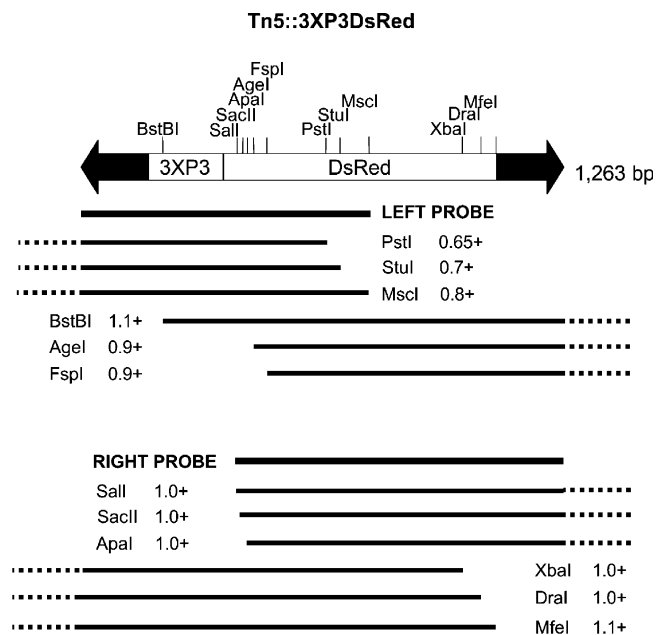


Fig. 2. Diagrammatic representation of *Tn5::3xP3DsRed* (not to scale). Filled, black arrows represent the terminal inverted repeats of *Tn5*. Restriction sites and the approximate size of a resulting junction fragment containing part of the *Tn5* vector and flanking genomic DNA are shown. The location of the sequences used as probes in Southern blot analysis are shown.

incubated at room temperature for 30 min in the absence of Mg^{2+} to create synaptic complexes (Fig. 1). The synaptic complexes were then diluted with water and injected. In the experiments described here, the final concentration of transposon DNA in the injection mixture ranged from 17 to 80 ng/ μ l.

2.2.3. Injection and post-injection procedures

Eggs were collected from *Ae. aegypti* females of the *kh^w* strain 3–5 days after blood-feeding, when daily egg production was greatest (*kh^w* was formerly referred to as *white eye*, Bhalla, 1968). Five to six adult females were isolated in Petri dishes lined with damp filter paper and placed in a dark incubator at 27 °C for approximately 30 min. Adults were removed and eggs were permitted to develop at 27 °C for an additional 30–45 min, at which time the chorions appeared light gray in color. Approximately 100 eggs were aligned on a piece of double-sided tape on a cover slip such that the embryonic anterior/posterior axes were aligned at approximately 45° to the edge of the cover slip (Fig. 3). The embryos were permitted to remain exposed to the air long enough for them to become partially dehydrated (approximately 2–3 min) at which time they were covered with Series 27 Halocarbon oil (Sigma). At 1–1.5 h post-oviposition, developing *Ae. aegypti* embryos consist of approximately eight cleavage nuclei located in the center of the egg (Raminani and Cupp, 1975) and to insure that the active complexes would rapidly encounter the chromosomes of the cleavage nuclei, the mixture was injected into the medial region of the developing embryo (Fig. 3). All injections were performed with a Pneumatic Pico Pump (World Precision Instruments) using beveled, quartz-glass needles (0.7 mm inside diameter) in conjunction with a dissecting microscope. Immediately following injection, the halocarbon oil was thoroughly drained from the embryos and the embryos were removed from the tape and placed on moist filter paper. Here, they developed for five days at room temperature and were then stimulated to hatch in a pan of deoxygenated water at 28 °C.

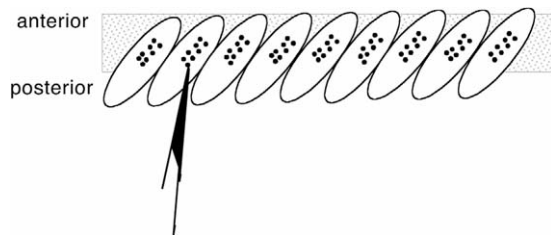


Fig. 3. Arrangement of embryos prior to injection. Eggs are immobilized on a glass slide using double-sided tape. Eggs are arranged so the needle can penetrate the side of the embryo, allowing some of the injection mixture to be deposited in the central ooplasmic space. At the time of injection, there are approximately eight cleavage nuclei located as shown.

Adult insects developing from injected embryos (G_0) were backcrossed to *kh^w*. Families consisting of approximately 10 G_0 males mated with 25 *kh^w* females and 10 G_0 females mated with 20 *kh^w* males were established. Progeny from these crosses (G_1) were screened for the presence of transgenic individuals by examining larvae and adults for *3xP3DsRed* expression in the brain and optic stalk using a Zeiss M²Bio equipped with an appropriate filter.

2.2.4. Transposable element display

Transposable element display is a DNA fingerprinting technique that permits the junction fragments consisting of known transposable element sequences and unknown flanking genomic DNA to be selectively amplified using PCR and visualized following electrophoresis (Casa et al., 2000). The methods used here were essentially those described by Guimond et al. (2003) with some modifications. Genomic DNA was isolated from individual adults according to the method (#48) described in Ashburner (1989), digested to completion with *MspI* and ligated to *Msp* adapters as described (Guimond et al., 2003). Preselective PCR reactions for analysis of the left- and right-end junction fragments were conducted using Tn5L-TEDA1 (5′GCT TGT TTG AAT TGA ATT GTC G3′) and Tn5R-TEDA1 (5′CTC TAG ATC ATA ATC AGC CAT ACC3′), respectively, in conjunction with the primer *MspIa* (Guimond et al., 2003). Preselective PCR conditions for left-end reactions began with a single cycle at 95 °C for 3 min; followed by 25 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 1 min and ending with a single cycle at 72 °C for 5 min. Preselective PCR conditions for right-end reactions began with a single cycle at 95 °C for 3 min; followed by 30 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 1 min and ending with a single cycle at 72 °C for 5 min. Selective PCR was conducted using Tn5L-TEDA2 (5′Cy5-ACG AAG CGC CTC TAT TTA TAC TCC3′) and Tn5R-TEDA2 (5′Cy5-CTG AAC CTG AAA CAT AAA ATG AAT GC3′) and primer *MspIa*. Selective PCR conditions for left-end reactions consisted of an initial cycle at 95 °C for 3 min; followed by five cycles of 95 °C for 15 s, an annealing temperature that was reduced 1 °C per cycle beginning at 69 °C for 30 s and an extension step at 72 °C for 1 min; followed by 25 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 1 min and ending with a single cycle at 72 °C for 5 min. Selective PCR conditions for right-end reactions consisted of an initial cycle at 95 °C for 3 min; followed by five cycles of 95 °C for 15 s, an annealing temperature that was reduced 1 °C per cycle beginning at 63 °C for 30 s and an extension step of 72 °C for 1 min; followed by 25 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 1 min and ending with a single cycle at 72 °C for 5 min. Selective PCR amplification products were

size fractionated on a 6% denaturing polyacrylamide DNA-sequencing gel at 70 W for 2.5 h. Gels were dried and scanned for red chemifluorescence on a STORM 860 phosphoimager (Molecular Dynamics). Gel images were reproduced to actual size on acetate film and overlaid on the dried gel to allow for precise location of bands of interest that were then excised. Excised bands were soaked in 50 μ l of dH₂O overnight and 5 μ l were used as a template in a selective PCR reaction with unlabeled selective PCR primers. Purified reamplification products were sequenced and BLAST searches conducted of the flanking DNA (Altschul et al., 1990).

2.2.5. Southern hybridization

Approximately 15 μ g of genomic DNA was digested to completion using a variety of enzymes under conditions recommended by the manufacturer (New England Biolabs). For Southern blots of genomic DNA probed with the left-end specific probe (Fig. 2), the DNA was digested with *Pst*I, *Stu*I, *Msc*I, *Bst*BI, *Age*I, *Fsp*I. For genomic Southern blots probed with the right-end specific probe (Fig. 2), the DNA was digested with *Sal*I, *Sac*II, *Apa*I, *Xba*I, *Dra*I and *Mfe*I. Digested DNA was size fractionated by electrophoresis in 1% agarose. Size-fractionated DNA was transferred to uncharged nylon filters (Duralon-UV[™], Stratagene) by capillary action overnight. DNA was covalently cross-linked using a Stratalinker (Stratagene), prehybridized in QuickHyb[®] (Stratagene) at 60 °C for 4–8 h and hybridized overnight in QuickHyb[®] with a ³²P-labelled probe. Probes were prepared by labeling PCR products produced using Tn5probeL-f (5'TTA TAC ACA TCT CAA CCA TCA TCG3') + Tn5probeL-r (5'CCA TGG TCT TCT TCT GCA TTA CG3') and Tn5probeR-f (5'CAC CAT GGT GCG CTC CTC C3') + Tn5probeR-r (5'TTA TAC ACA TCT CAA CCC TGA AGC3') using pTn5::3xP3DsRed as a template. Reactions consisted of 95 °C for 3 min; followed by 30 cycles of 95 °C for 15 s, 65 °C for 30 s and 72 °C for 1 min; and 72 °C for 5 min. Amplification products were gel purified and their concentration determined.

Amplification products (25 ng) were labeled with ³²P by the random priming method according to the manufacturer's recommendations (Prime-It[®] II, Stratagene). Hybridized filters were washed three times in 2 \times SSC + 0.1% SDS for 30 min at room temperature, and twice in 0.1 \times SSC + 0.1% SDS for 30 min at 50 °C. Hybridization signals were detected using a Storm 860 phosphoimager (Molecular Dynamics).

3. Results

3.1. Tn5 activity in *Ae. aegypti* embryos

Following the injection of a mixture of the EZ::TN[™]<KAN>Tnp Transposome[™] and the target plasmid pUCSacRB into pre-blastoderm embryos, recombinant target plasmids with integrated EZ::TN[™]<KAN> elements were recovered (Table 1). Of the 2 \times 10⁴ target plasmids recovered and screened from injected embryos, 24 were recombinants arising from integration of the EZ::TN[™]<KAN> element into the *SacRB* gene, resulting in resistance of *E. coli* expressing this gene to sucrose. Recombination (transposition) only occurred in developing embryos and not in the injection mixture prior to injection because transforming *E. coli* directly with the injection mixture did not result in any recombinant target plasmids being recovered after screening more than 1 \times 10⁵ plasmids. The frequency of recombinants resulting in a loss of activity of the *SacRB* target gene in embryos was approximately the same (1.2 \times 10⁻³) as that observed when EZ::TN[™]<KAN>Tnp Transposome[™] transposition was measured in vitro (1 \times 10⁻³; Table 1). Fifteen recombinant target plasmids, all of which appeared to contain a single *Tn5* element, based on diagnostic restriction endonuclease digestion (not shown), were analyzed further by determining the DNA sequence of the insertion sites. Nine of the recombinants analyzed resulted in the perfect integration of the EZ::TN[™]<KAN> element with a 9 bp direct duplication at the point of integration, as is

Table 1
Tn5 mobility in *Aedes* embryos

Conditions	Targets screened	Transpositions ^a
Injection mix only ^b	> 100 \times 10 ³	0
Injection mix + embryos	20 \times 10 ³	24
In vitro reaction ^c	5 \times 10 ³	5

^a Transpositions refer to the recovery of recombinant pUCSac plasmids that now contain *Tn5::Kan*. This resulted in plasmids that conferred ampicillin, kanamycin and sucrose resistance. All plasmids thought to contain a transposition event were confirmed by appropriate restriction endonuclease digestion and mapping.

^b The injection cocktail consisted of 4.0 μ l of water containing 0.5 μ g/ μ l of pUCSac and 1 μ l of EZ::TN<KAN-1>Tnp Transposome[™] (Epicentre Technologies). One microliter of this cocktail was used to transform *E. coli* directly.

^c The in vitro reaction was conducted according to the manufactures recommendations.

typical for *Tn5*. The remaining six recombinants were non-canonical integration events, in that 9 bp target site duplications were not seen and sequences flanking the left and right ITRs were not, in some cases, contiguous in the original target plasmid. All these events resulted from the *EZ::TN[™]<KAN>* element integrating into the *SacRB* target gene as reflected in the elimination of sucrose sensitivity and the presence of *SacRB* sequences flanking at least one of the ITRs of the element. Four of the recovered transposition events had pUC19 DNA sequences of the target plasmid flanking the right ITR of *EZ::TN[™]<KAN>* suggesting that integration was accompanied by either DNA deletion or rearrangement. In two of the integration events, the *EZ::TN[™]<KAN>* element had lost the terminal nucleotide (Table 2).

The nine remaining recombinants arising from integration of *EZ::TN[™]<KAN>* had multiple elements based on diagnostic restriction endonuclease digestion. These were not analyzed further.

3.2. *Tn5* as a germ-line transformation vector

905 G_0 adult mosquitoes were produced by injecting approximately 6000 pre-blastoderm embryos with pre-assembled *Tn5::3xP3DsRed* synaptic complexes. Families were established consisting of 10 G_0 s of the same sex and approximately twice as many *kh^w* of the opposite sex, as described in Materials and methods. Two families yielded G_1 progeny with *3xP3DsRed* expression in the brain and optic stalks, for an estimated frequency of transformation of 0.22%. (Assuming a single G_0 in each family produced the transgenics and that all G_0 s were fertile, the transformation frequency is $2/905 = 0.22\%$.) This is an under-

estimate of the transformation frequency because usually there are G_0 s that are sterile as a result of the injection procedure. In this experiment, however, we injected the embryos medially and avoided damaging the posterior pole of the embryo, and therefore expected less injection-induced sterility than in previous *Ae. aegypti* transformation efforts. Family 35 yielded a single transgenic progeny, while family 50 yielded 27 transgenic progeny. The phenotypes of the transgenic insects were essentially the same as has been described for transgenic *Ae. aegypti* expressing *3xP3::EGFP* (Kokoza et al., 2001; Wilson et al., 2003) except with red instead of green fluorescence. The phenotypes of all transgenics consisted of red-fluorescent brains and optic stalks in larval, pupal and adult stages. The transgenic progeny from family 50 consisted of individuals with two distinguishable phenotypes, one with bright red fluorescence in the eyes of adults and the other with distinctly less bright fluorescence in the eyes. Individuals from family 50 of each phenotype were used to start separate lines, 50a (light) and 50b (bright). A single line was established from the transgenic progeny arising from family 35. The three lines were created by initially crossing transgenics to *kh^w* individuals and then repeatedly selecting and interbreeding transgenics at each generation.

Evidence for the physical integration of the *Tn5::3xP3DsRed* element was obtained from Southern hybridization and transposon display experiments. The patterns of hybridization observed following Southern blot analysis of Line 35 at G_4 showed multiple (approximately 5), strongly hybridizing bands when the left end- and right end-specific probes were used. The patterns of bands observed were similar for each of the enzymes used to digest the genomic DNA (Figs. 2

Table 2
Tn5 integration site analysis in insect cells

No.	Sequence	Right	<i>Tn5</i>	Left	Sequence
<i>Canonical Insertion events</i>					
1	<i>SacRB</i>	aaacaCTGAAAAC		CTGAAAACgcaaa	<i>SacRB</i>
2	<i>SacRB</i>	tagtgaGGATATCTC		GGATATCTCagcgta	<i>SacRB</i>
3	<i>SacRB</i>	caaagaCGATGTGGT		CGATGTGGTagecgt	<i>SacRB</i>
4	<i>SacRB</i>	attaacCTTTACTAC		CTTTACTACcgcaact	<i>SacRB</i>
7	<i>SacRB</i>	gcgcctCCTGCCAGC		CCTGCCAGCagtgcg	<i>SacRB</i>
11	<i>SacRB</i>	aacgatATTTAAATG		ATTTAAATGcttgg	<i>SacRB</i>
16	<i>SacRB</i>	agtaaaGGTTAATAC		GGTTAATACgttgc	<i>SacRB</i>
18	<i>SacRB</i>	tgtggaAGCCGTGAT		AGCCGTGATagttg	<i>SacRB</i>
19	<i>SacRB</i>	gcaaaaGGCCTGGAC		GGCCTGGACgtttgg	<i>SacRB</i>
<i>Non-canonical insertion events</i>					
6	<i>pUC19</i>	cgactcTGCATCCTC		AGATGTGCTaatcct	<i>SacRB</i>
14	<i>pUC19</i>	tctatcCTAAAAGAC		CGGGGATCAatgaac	<i>SacRB</i>
17	<i>pUC19</i>	ttacgaTTTTCTGC		CTGGCATTAAatgaat	<i>SacRB</i>
20	<i>SacRB</i>	aaacacTGAAAAC		CTGACAACTacacaa	<i>SacRB</i>
21	<i>pUC19</i>	gaggttGTGTCAGGA	ΔC	TACGCGTAAaacagc	<i>SacRB</i>
24	<i>SacRB</i>	attatcATGCTTGGT		CCTGAGACAtctgac	<i>SacRB</i>

and 4). All hybridizing bands were tightly clustered and ranged in size from approximately 2.5 to 0.6 kb and hybridizing bands. Lines 50a and 50b, despite their phenotypic differences, had indistinguishable patterns of hybridization. The patterns of hybridization were much simpler than that seen with Line 35, and for each enzymatic digestion of genomic DNA for Lines 50a and 50b, a distinct pattern of two or three hybridizing bands was observed.

Transposable element display was performed after G₂ and G₄, and produced results that were consistent with those obtained from the hybridization studies.

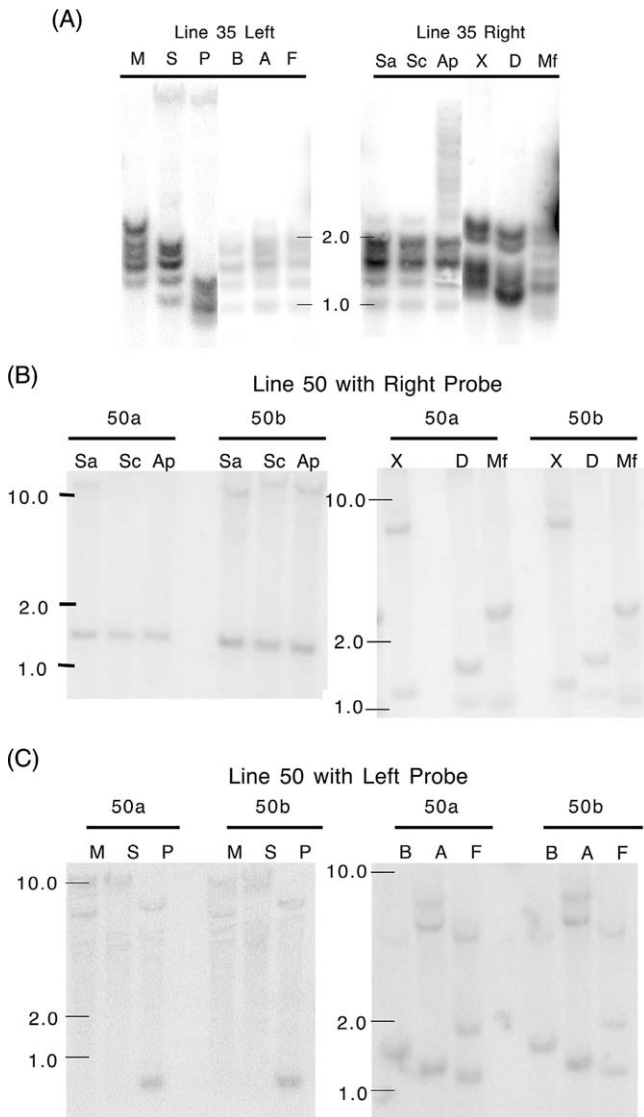


Fig. 4. Southern blot analysis of transgenic lines at generation 4 (G₄). (A) Analysis of Line 35 using the left- and right-end probes (see Fig. 1); (B) Analysis of Lines 50a and 50b using the right-end probe; (C) Analysis of Lines 50a and 50b using the left-end probe; A, AgeI; Ap, ApaI; B, BstBI; D, DraI; F, FspI; M, MscI; Mf, MfeI; S, StuI; Sa, SalI; Sc, SacI; X, XbaI. Numbers with bars indicate the size (in kilobasepairs) and position of a molecular weight marker.

Transposable element display of individuals from Lines 50 and 35 at G₂ revealed evidence of at least 5 and 7 elements, respectively. In Line 35, all the detected elements were present in all individuals genotyped, suggesting close linkage and the pattern of bands was identical to that observed at G₄. Individuals from Line 50 at G₂, however, contained from one to three elements but there was no evidence for linkage between any of the elements (Fig. 5). Repeating the analysis after G₄ revealed no change in the pattern of bands in Line 35, however, the pattern in Lines 50a and 50b were different from that observed at G₂. While Lines 50a and 50b were indistinguishable from each other based on TE display, some elements displayed at G₂ were absent (or were at low frequencies within the population) at G₄ (Fig. 5). The elements present in Lines 50a and 50b at G₄ appeared linked since all individuals tested had the same genotype. Close linkage of all elements present in the lines at G₄ was also demonstrated by the pattern of inheritance of the red fluorescence phenotype associated with the expression of *3xP3DsRed*. Approximately 50% of the progeny arising from crosses between *kh^w* individuals and individuals

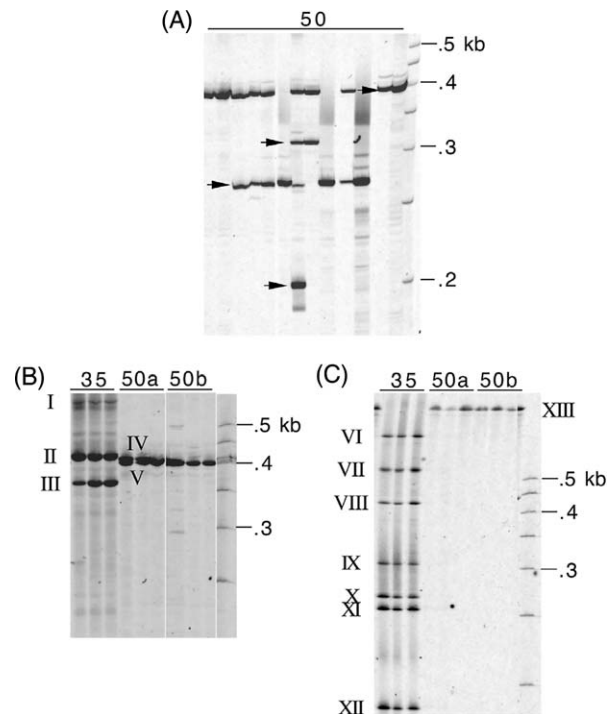


Fig. 5. Transposable element display of transgenic lines. (A) Line 50 at G₂. Each lane is the analysis of the genomic DNA from a single individual; (B) Transposable element display of right-end junction fragments at G₄. The analysis of three individuals from Lines 35, 50a and 50b are shown; (C) Transposable element display of left-end junction fragments at G₄. Analysis is otherwise like that in (B). Each junction fragment was labeled I–XIII. Positions of molecular weight size standards are shown.

from Lines 35 and 50 had *3xP3DsRed* expression in the brain and eyes (data not shown).

Isolation, reamplification and DNA sequence analysis of some of the bands obtained at G₄ revealed that in some cases, the termini of *Tn5::3xP3DsRed* were now adjacent to either sequences found inside *Tn5::3xP3DsRed* or the termini of other *Tn5::3xP3DsRed* elements (Fig. 7). In addition to evidence for nested and tandemly arranged elements, junction fragments were recovered that had the right and left ITR adjacent to *Ae. aegypti* genomic DNA. BLAST searches did not reveal any significant similarity to any sequences within existing databases (as of June 2003). Four of the termini recovered from TE display had evidence of small deletions of between 3 and 12 of the most distal nucleotides.

4. Discussion

The results of the experiments reported here indicate that *Tn5* can function in insects. Transient *in vivo* mobility assays in which assembled synaptic complexes were co-injected with a target plasmid into the ooplasm of young developing mosquito embryos resulted in the generation of recombinant target plasmids containing a copy of the *Tn5* element. These types of transposable element mobility assays have been routinely used to assess the functionality of Class II transposable elements in various insect systems (Atkinson et al., 2001). Although these types of experiments intend to assess the mobility of the element in somatic cells, they also have been good indicators of an element's ability to serve as a germ-line transformation vector. In previous experiments of this type, the frequency of recombinant target plasmid recovery was 10^{-3} – 10^{-5} . *Mos1*, *Hermes* and *piggyBac* transpose in *Ae. aegypti* embryos at frequencies of 10^{-5} , 3×10^{-5} and 3×10^{-3} , respectively, and *Minos* transposes in *Ae. aegypti* cell lines at a frequency of 10^{-5} (Coates et al., 1998; Klinakis et al., 2000; Lobo et al., 1999; Sarkar et al., 1997). *Mos1*, *Hermes* and *piggyBac* have been shown to function as germ-line transformation vectors in *Ae. aegypti* with similar efficiencies, resulting in transformation rates of approximately 4%, 11% and 4%, respectively (Coates et al., 1998; Jasinskiene et al., 1998; Lobo et al., 2002). Here, we observed a frequency of *Tn5* transposition of 1.2×10^{-3} that was similar to and, in some cases, considerably higher than that observed with current insect gene vectors. These data suggested that the direct injection of pre-assembled synaptic complexes of *Tn5* might provide an alternative method for creating transgenic insects, and that *Tn5* may be at least equally efficient as existing germ-line transformation vectors in insects. An unusual aspect of the *Tn5* system as revealed by the analysis of the actual sites of integration was the non-

canonical nature of some of the integration events. That is, the process of integration of *Tn5* typically results in the creation a 9 bp direct duplication of the target site with one copy of the duplication flanking the right and left IRTs of the element. In the collection of 15 *EZ::TN[®]<KAN>* integration events recovered in this experiment and analyzed in some detail, only nine resembled canonical integration events with 9 bp direct duplications flanking the left and right ends of the element. The remaining integration events were unusual in that they lacked a direct duplication at the site of integration, and the sequences flanking the element were in different locations within the target plasmid. Mapping of the recombinant target plasmids indicated that each plasmid included in this analysis contained a single *EZ::TN[®]<KAN>* element and that the size of the recombinant plasmid was approximately as expected. Integration in these cases appears to be accompanied by deletions of target plasmid sequences.

It was anticipated that synaptic complexes would be short-lived and that any integrations that do occur would occur shortly after injection when there are approximately 20 nuclei. Thus, the resulting G₀ will be a mosaic consisting of a substantial number (5–10%) of transgenic cells. Such a level of mosaicism should be reflected in the number of transgenic progeny produced by the mosaic G₀ and integrations early in development are expected to produce large clusters of transgenic progeny. That is, a large number of progeny from a transgenic (mosaic) G₀ are expected to be transgenic. In this experiment, family 50 consisted of 6 G₀s and produced approximately 600 progeny of which 27 were transgenic. If each G₀ produced approximately 100 progeny and the transgenics arose in the germ-line of a single G₀ individual, then the 27 transgenic progeny recovered represent a large cluster (27/100). On the other hand, family 35 also consisted of 6 G₀ individuals that produced approximately 100 progeny each. However, only a single transgenic progeny was recovered, indicating that the cluster size in this case was quite small (1%). Line 35 had no obvious problems with viability and appeared to have an average fertility and fecundity, indicating that the transgenic event did not result in partial sterility or lethality. Cluster size reflects, to some extent, the timing of the transgenic event, with early events leading to larger clusters. The low number of transgenic progeny produced by family 35 suggests that the integration event occurred after the establishment of the germ-line, 3–4 h after egg deposition. The event recovered in Line 35 suggests that the half-life of *Tn5* synaptic complexes may not be short-lived in embryos, as originally assumed.

In the transgenic lines analyzed in this experiment, all contained multiple *Tn5* elements. Initial investigation of Line 50 at G₂ by transposable element display revealed the presence of at least four unlinked

elements. Analysis at G₄ of Lines 50a and 50b which were derived from Line 50, revealed only two elements and indicated that some of the elements detected at G₂ had been lost either as the result of instability or genetic drift. All molecular analyses indicate that Lines 50a and 50b are identical. Line 35 appeared to contain approximately five to seven elements. Transposable element display of the left and right ends of *Tn5::3xP3DsRed* produced results reflecting different numbers of elements. Left-end analysis indicated the presence of seven elements, while right-end analysis indicated the presence of only three elements. Southern hybridization analysis of Line 35 indicated the presence of five elements. The difference in estimates of element numbers based on the results of transposable element display of right and left ITRs may either reflect the absence of right ITRs or indicate that the transposable element display method failed to detect the ends that were present. Transposable element display tends to undercount elements since only those fragments efficiently amplified under the conditions chosen (1 kb or less) can be visualized. If an ITR is contained on a fragment greater than 1 kb, it might be missed in this analysis.

All the elements in Lines 35 and 50 appeared to be associated with each other and exist as either tandem or nested insertions, where copies of *Tn5::3xP3DsRed* were integrated either into or next to other copies of *Tn5::3xP3DsRed*. This was seen most clearly in the

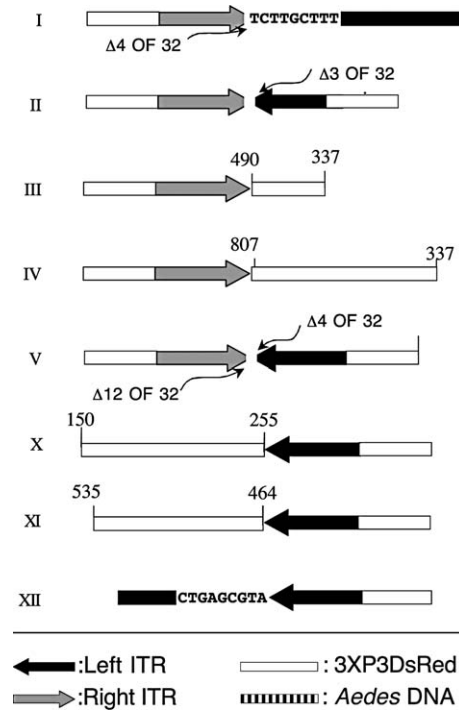


Fig. 6. DNA sequence analysis of junction fragments. Fragment numbers correspond to those in Fig. 5. Notable features of each fragment are labeled including locations of left and right ITRs; *3xP3DsRed* transgene; *Ae. aegypti* genomic DNA; position and size of deletions at the termini of the ITRs. Numbers refer to nucleotide positions within *Tn5::3xP3DsRed* beginning with the terminal nucleotide of the left ITR.

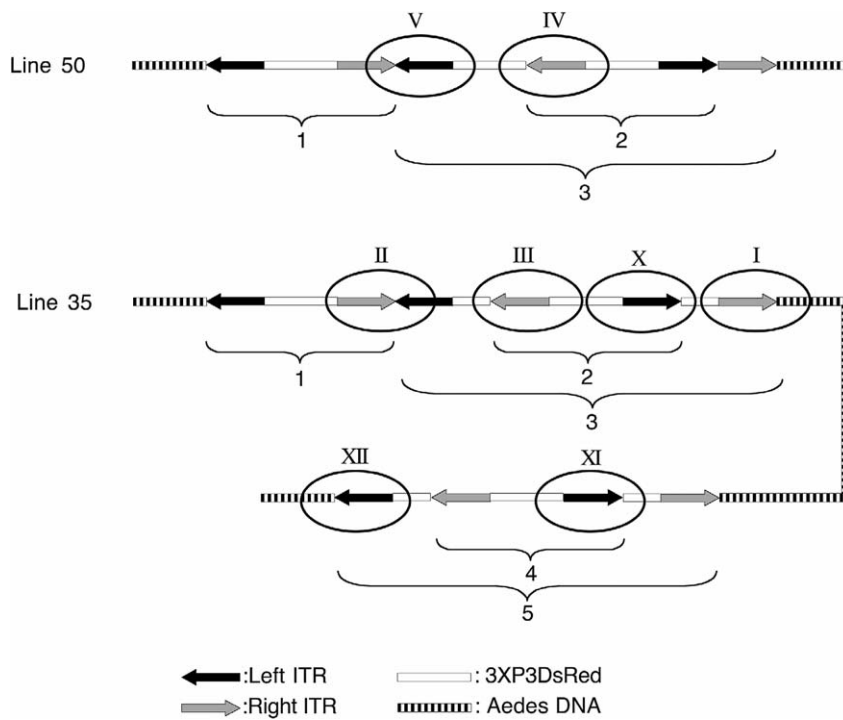


Fig. 7. Models of integration events present in Lines 35 and 50. Brackets indicate the position of individual *Tn5::3xP3DsRed* elements. Ovals indicate junction fragments for which DNA sequence data have been obtained. Roman numerals correspond to junction fragments referred to in Fig. 6.

results of transposable element display in which sequence analysis showed the presence of the *Tn5* terminal sequences inserted into sequences present in the vector (nested integration) or immediately adjacent to the terminal sequences of another *Tn5* element (tandem insertion; Fig. 6). Nested and tandem insertions of *Tn5* tended to explain the very regular and almost invariant pattern of hybridization observed during Southern analysis of Line 35 using different enzymes. Southern analysis was performed using restriction endonucleases that cut only once within the *Tn5::3xP3DsRed* element and probes were chosen to hybridize to the resulting junction fragments. For each enzyme used, the junction fragment is expected to be a different size based on the location of the appropriate restriction site in the flanking genomic DNA. The pattern of junction fragments obtained for Line 35 indicated that all the restriction sites in the flanking DNA were clustered, resulting in the pattern of fragments seen. It is unlikely that the six-base restriction sites chosen would be clustered in the flanking genomic DNA just as they were in the element. Instead, the clustered restriction sites flanking the elements are more likely to be actually in other copies of the element. Fig. 7 illustrates hypothetical models of the Lines 35 and 50 integration events. The models capture a number of the results of this analysis including copy number estimates, transposable element display results and some of the Southern analysis data. The models capture key characteristics of *Tn5::3xP3DsRed* integration in *Ae. aegypti* including multiply linked and nested elements.

The results of this study show that *Tn5* is functional in insect cells and can be used to create transgenic insects. The relatively low rate of transformation and the complex arrangement of multiple elements that occurs during transformation tend to make this system less useful than the other insect vector systems currently available that are constructed from insect Class II transposable elements. However, the results obtained in this experiment are unlikely to represent the optimal behavior of this element in insects. Indeed, the problematic performance of this system in insects may be due to its hyperactivity and our lack of familiarity with it. We feel that the concentration of synaptic complexes injected is a critical variable and injecting high concentrations of synaptic complexes may be counter-productive. Because pre-assembled synaptic complexes become active in the presence of Mg^{2+} , as soon as the complexes enter the ooplasm they are activated. Active complexes will then integrate into abundantly available target DNA. The most abundant targets at the time of injection may be other copies of *Tn5::3xP3DsRed*. Instead of integrating into chromosomal DNA, which is at a low concentration relative to the *Tn5* vector, the complexes integrate into each other. This might account for the complex nested integrations seen in

Lines 35 and 50. To avoid this problem, one may need to significantly reduce the concentration of injected synaptic complexes. The limitations of the *Tn5* system as it was used here may reflect general limitations to the use of active intermediates as starting points in a transformation experiment. While most transposons assembled into active synaptic complexes may suffer from the problem of self-integration when used as described here, it will be worth testing existing insect vectors in this way as part of an effort to improve transformation rates.

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