

Green fluorescent protein as a genetic marker in transgenic *Aedes aegypti*

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Abstract

We report here the use of the enhanced green fluorescent protein (EGFP) from the jellyfish, *Aequorea victoria*, as a genetic marker for the genetic transformation of mosquitoes. The EGFP gene, under the control of the actin5C promoter of *Drosophila melanogaster* was inserted into the *Hermes* transposable element. Preblastoderm embryos of a wild-type strain of the yellow fever mosquito, *Aedes aegypti*, were microinjected with this plasmid, together with a helper plasmid containing the *Hermes* transposase placed under the control of the *D. melanogaster hsp70* promoter. Somatic EGFP expression was observed during early instars in approximately one-half of all G₀ individuals. Two G₁ individuals arising from a G₀ female displayed high levels of EGFP gene expression during all stages of development. EGFP was transmitted in a Mendelian fashion to the G₂ and G₃ generations and molecular analysis confirmed the presence of the *Hermes*[actin5C:EGFP] gene in these insects. These results clearly demonstrate that EGFP can be used as an effective genetic marker in wild-type *Ae. aegypti* and most likely in other mosquito species as well.

Keywords: *Aedes aegypti*, transformation, *Hermes* transposable element, green fluorescent protein.

Introduction

A major problem in producing and recognizing transgenic

insects in species other than the vinegar fly, *Drosophila melanogaster*, has been the lack of available genetic markers that produce unequivocal phenotypes. A transformed line of *Anopheles gambiae* was generated using a plasmid that contained a *P* element into which the bacterial neomycin phosphotransferase gene conferring resistance to the antibiotic G418 had been inserted (Miller *et al.*, 1987). However, in general, selection for resistance to G418 in insects can be extremely problematic. The use of an antibiotic or insecticide resistance strategy to select transgenic insects can lead to the generation of false positives due to, for example, breakdown of the insecticide or antibiotic. Furthermore, genuine transformants can be lost if the level of expression of the transgene is not sufficient to confer antibiotic or insecticide resistance to the transgenic individual. More recently, the identification of genetic transformants of the yellow fever mosquito, *Aedes aegypti*, and the Mediterranean fruit fly, *Ceratitis capitata*, was facilitated by using specific eye pigmentation genes as the genetic marker (Loukeris *et al.*, 1995; Coates *et al.*, 1998; Handler *et al.*, 1998; Jasinskiene *et al.*, 1998; K. Michel, D. A. O'Brochta, A. C. Pinkerton, G. Franz, A. S. Robinson, P. W. Atkinson, unpublished data). A *D. melanogaster* genomic clone of the *cinnabar* gene was placed into either *Hermes* (Jasinskiene *et al.*, 1998) or *mariner* (Coates *et al.*, 1997) transposable elements and these transposable elements were then used to genetically transform an *Ae. aegypti* mutant (*kh^w*), deficient in the enzyme kynurenine 3-hydroxylase – the gene product of the *cinnabar* mutation of *D. melanogaster* (Cornel *et al.*, 1998). Similarly, a cDNA clone of the *white* gene of *C. capitata* was placed into the *Minos*, *piggyBac* and *Hermes* elements and each of these transposable elements was used to genetically transform a *white* (*w*) mutant of *C. capitata* (Loukeris *et al.*, 1995; Handler *et al.*, 1998; K. Michel, D. A. O'Brochta, A. C. Pinkerton, G. Franz, A. S. Robinson, P. W. Atkinson, unpublished data).

To date only a limited number of non-drosophilid insect species have been transformed using the four transposable element vectors, *Minos*, *Mos1*, *Hermes* and *piggyBac* now available. It is perhaps not surprising that the recent successes in developing these transgenic systems have occurred in two insect species in which eye pigmentation mutants exist, each of which can be complemented by previously

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cloned genes. However, amongst insects, the availability of such mutations and the corresponding cloned genes is, at best, scarce. Furthermore, there is strong evidence from transposable element mobility assays that the *Hermes*, *mariner* and *piggyBac* elements can accurately transpose in a range of medically and agriculturally important insect species in which no suitable mutations exist (Pinkerton *et al.*, 1996; Coates *et al.*, 1997; Sarkar *et al.*, 1997a,b; Thibault *et al.*, 1999).

This lack of visible mutants and corresponding cloned genes might be predicted to be the next hurdle facing insect scientists and parasitologists who seek to introduce genes into these insect species. This paper reports the use of the enhanced form of the green fluorescent protein (EGFP) gene as a genetic marker to recognize transgenic lines of *Ae. aegypti*. The GFP gene has, over the past few years, been used as a genetic marker to enable the identification of transgenic lines in organisms as diverse as *D. melanogaster* (Plautz *et al.*, 1996), *Caenorhabditis elegans* (Chalfie *et al.*, 1994), *Danio rerio* (Amsterdam *et al.*, 1995) and mammals (Ikawa *et al.*, 1999). In addition, the GFP gene has been shown to function as a reporter gene in mosquitoes infected with Sindbis virus expressing GFP, indicating that this gene should be an effective genetic marker in transgenic mosquitoes (Higgs *et al.*, 1996). The data presented here demonstrate that the GFP gene can be used as a genetic marker that easily permits the identification of transgenic *Ae. aegypti* and suggest that this gene should also prove effective in the development of transgenic strategies for other mosquito species.

Results

Enhanced green fluorescent protein as a primary genetic marker in Drosophila melanogaster

A *Hermes* element containing the EGFP gene placed under the control of the promoter proximal to exon 1 of the actin 5C promoter of *D. melanogaster* was constructed (see Experimental procedures) and coinjected with a *Hermes* helper plasmid into preblastoderm *D. melanogaster* embryos. The *D. melanogaster* strain used for these experiments was a *yw*^{67c23} strain and did not contain any full-length *hobo* elements. *Hobo* elements, like *Hermes*, are members of the *hAT* element family of transposable elements and strains in which *hobo* elements are absent ('E' strains) are used as recipients in genetic transformation experiments using *hobo* as the transposable element vector (Blackman *et al.*, 1989). This strain has previously been used as the recipient strain in *Hermes* transformation experiments conducted in our laboratory in which the *white* gene of *D. melanogaster* was used as the primary genetic marker (O'Brochta *et al.*, 1996).

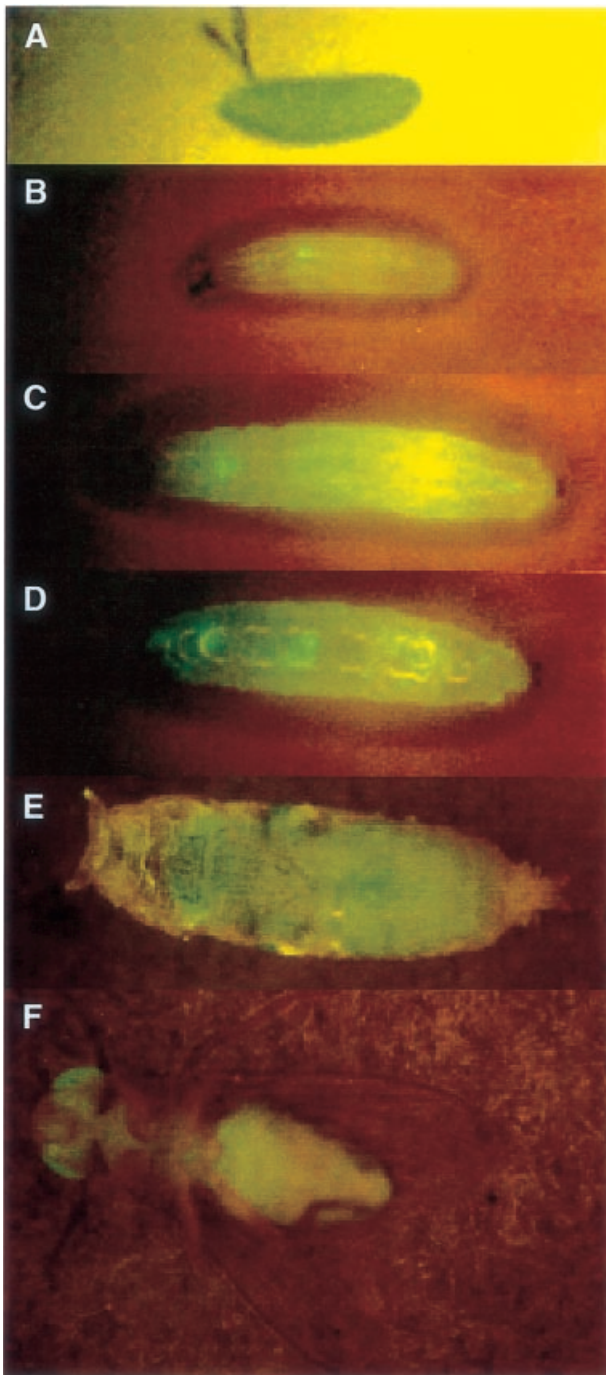
A total of 250 embryos were injected and fourteen fertile G₀ matings were obtained. From these, three matings

produced transgenic offspring expressing the EGFP gene giving a transformation frequency of 21.3%. This frequency is consistent with the *Hermes* element transformation frequencies previously reported for *D. melanogaster* (O'Brochta *et al.*, 1996). Significantly, G₁ transformants expressing EGFP were easily recognized at all life stages, from embryo to adult, further demonstrating the effectiveness of the EGFP gene as a genetic marker in *D. melanogaster*. The results of these experiments are shown in Fig. 1 (A–F). EGFP expression is clearly obvious in embryos and larvae (Fig. 1A–D). Strongest expression in late-stage embryos was seen in sections of the gut, a result that is expected when using the exon 1 proximal promoter of the actin5C gene (Burn *et al.*, 1989). Expression of EGFP was very clear in pupae (Fig. 1E), consistent with there being an increase in cell division during this life stage. Expression in adults varied between strong expression throughout the entire animal to individual lines in which expression was strongest in the gonads (Fig. 1F). These differences were most likely due to position effect between the three lines with, in the later case, the expression of the transgene being reduced except in those tissues in which there is a high level of cell division.

The EGFP gene was inherited in a Mendelian fashion in subsequent generations, consistent with a single integration event in each of the three lines generated. Molecular analysis of the integrated DNA was performed using inverse polymerase chain reaction (IPCR) and confirmed that integration of the *Hermes*[act5C:EGFP] element had occurred by transpositional recombination (Fig. 1G). The sequences integrated into the *D. melanogaster* genome were delimited by the 17 bp inverted terminal repeats (ITRs) of the *Hermes* element and an 8 bp target site duplication was created at each target site. The consensus sequence, 5'-AtnGnnnC-3', of this duplicated sequence was consistent with the DNA sequence of target site duplications created by transposition of the *Hermes* element in previous experiments (O'Brochta *et al.*, 1996; Pinkerton *et al.*, 1996; Sarkar *et al.*, 1997a,b). In line F1–2, comparison of the flanking sequences to the Berkeley *Drosophila* Genome Project database showed that the *Hermes* element had inserted into a region of the genome located on chromosome 2R between 56F8 and 56F12 and contained within the P1 clone DS08872 located in the contig mus209. Line M1–2 had inserted into a sequence represented in the *D. melanogaster* Expressed Sequence Tag (EST) clone LD28878.5'.

Green fluorescent protein as a primary genetic marker in Aedes aegypti

The success of using EGFP as a primary marker for *D. melanogaster* transformation led us to examine whether this marker alone could be used to identify *Ae. aegypti* transgenic lines. Newly laid wild-type *Ae. aegypti* eggs were coinjected with the same plasmids used for the *D. melanogaster* transformation experiments according to standard procedures



G

Line	Target site sequence
M1-1:	5'-ATAGCCAC-3'
M1-2:	5'-ATGTGTAC-3'
F1-2:	5'-ATTGAAAT-3'
Consensus:	5'-ATnGnnnAC-3'

Figure 1. (A–F) Transgenic line of *D. melanogaster* containing the Hermes[act5C:EGFP] element showing expression of EGFP during the various life stages. (A) Embryo; (B) first-instar larva; (C) second-instar larva; (D) third-instar larva; (E) pupa; (F) adult; (G) target site duplications present at the integration sites of the Hermes[act5C:EGFP] element in *D. melanogaster*.

(Jasinskiene *et al.*, 1998). A total of fifty-three (thirty-one males, twenty-two females) living first-instar G_0 adult mosquitoes were obtained following microinjection of approximately 100 embryos. Approximately one-half of all G_0 larvae arising from injected embryos displayed varying levels of green fluorescence, typically seen as small spots of fluorescence in various regions of the larvae. No fluorescence was observed in the pupae or adults of any of the G_0 mosquitoes examined. A total of twenty-three families (that is a G_0 male \times four or five females) and three pool matings (that is a total of twenty G_0 females \times twenty to twenty-five males) were established and produced G_1 progeny. These were then scored for green fluorescence at the first-instar stage over two or three separate ovipositions. The results from these crosses are shown in Table 1.

None of the individual matings to G_0 males produced any offspring that exhibited green fluorescence. A faint yellow–green fluorescence was observed in the gut of most of the larvae. We believe this is caused by some undigested component of the diet displaying a faint yellow–green fluorescence under the excitation and detection conditions used. Two G_1 larvae from the third of the pool matings with G_0 females had a distinct, bright-green fluorescence surrounding their gut, gastric caeca and tissue surrounding the gut. Dissections of larvae from subsequent generations showed that the highest levels of EGFP were found in the fat body which is associated with the gut in the intact larvae. Lower levels of EGFP were observed in the gut and in the head. In undissected larvae, detection of EGFP expression in the head was obscured by the cuticle.

These two G_1 individuals were easily and instantly discernible from their siblings (of which there were approximately 250 in each watch glass) when they were initially examined in a Syracuse watch glass under the microscopy conditions used. Assuming these two individuals were genetic transformants containing the Hermes[act5C:EGFP] element and, assuming they were daughters of the same G_0 female, the transformation frequency for this experiment would be between 2.3 and 3.8% which is consistent with the *Hermes* and *Mos1* transformation frequency of *Ae. aegypti* reported by Jasinskiene *et al.* (1998) and Coates *et al.* (1998). The precise figure cannot be determined since the use of pool matings precludes an accurate determination of how many G_0 females are actually fertile.

These two green fluorescent individuals were separated from most of their siblings and allowed to develop separately from each other through the larval instars but with five or six non-transformed siblings. Their strong green fluorescence was sustained throughout all stages of larval and pupal development. These fluorescent pupae were then separated from the non-fluorescent siblings. Upon emergence, green fluorescence in the adults was located mainly towards the anterior or posterior of the abdomen, a phenotype we have previously noted with some transgenic *D. melanogaster* strains

Table 1. Results of injections of pHermes[act5C:EGFP] and pHSHH14 into *Ae. aegypti* embryos

Experiment No.	No. of G ₀ adults	No. of fertile matings	No. of G ₁ larvae scored	No. of EGFP ⁺ G ₁ larvae
1	11 ♂	9	2109	0
	6 ♀	Pool 1 (6 ♂)		0
2	20 ♂	14	2827	0
	16 ♀	Pool 2 (8 ♀)		0
		Pool 3 (6 ♀)		2
Total	53	26 < n < 43	4936	2

Table 2. Inheritance of the EGFP gene in the G₂ generation of *Ae. aegypti*

	EGFP ⁺ ♂ × 4 non-GFP ♀ sibs		EGFP ⁺ ♀ × 4 non-GFP ♂ sibs	
	EGFP ⁺	EGFP ⁻	EGFP ⁺	EGFP ⁻
1st oviposition	111	107	27	30
2nd oviposition	117	125	31	30
3rd oviposition	—	—	15	15
Total	228	232	73	75
Expected	230	230	74	74
	$\chi^2 = 3.48 \times 10^{-2}$ $P = 0.90-0.95$		$\chi^2 = 2.70 \times 10^{-2}$ $P = 0.90-0.95$	

containing the same transposable element. A single G₁ female and a single G₁ male were obtained and there was no discernible difference in the pattern of fluorescence between them.

These two fluorescent G₁ adults were then out-crossed with non-fluorescent adults and the G₂ first-instar larvae scored for fluorescence. Both matings were fertile and eggs were obtained from multiple ovipositions from both matings. The G₂ generation was examined for the presence or absence of the expression of the EGFP gene. As predicted for the Mendelian inheritance of a single gene, there was a 1 : 1 ratio of EGFP⁺ and EGFP⁻ G₂ individuals in both lines (Table 2). This was consistent with the insertion of the Hermes[act5C:EGFP] transposon at a single location in the *Ae. aegypti* genome.

Within each line, the EGFP⁺ G₂ individuals were self crossed and the inheritance and expression of the EGFP gene examined in the G₃ generation. The expectation from the this cross (in which all G₂ EGFP⁺ parents should be heterozygous for the EGFP gene) would be a 3 : 1 ratio of EGFP⁺ : EGFP⁻ individuals in the G₃ generation. This was observed in both lines. For the line derived from the G₁ EGFP⁺ female, 100 G₃ progeny were EGFP⁺ and 40 were EGFP⁻ ($\chi^2 = 2.7 \times 10^{-2}$, $P = 0.30-0.50$). For the line derived from the G₁ EGFP⁺ male, 228 G₃ progeny were EGFP⁺ and eighty-two were EGFP⁻ ($\chi^2 = 3.48 \times 10^{-2}$, $P = 0.50-0.70$). Within the EGFP⁺ class of the G₃ generation, one-third would be expected to be homozygous for the EGFP gene. The question of whether these homozygous individuals could be discerned from heterozygotes based on the relat-

ive level of green fluorescence was examined. From each G₂ mating, there appeared to be two levels of green fluorescence observed in those G₃ progeny that contained the EGFP gene. The ratio of putative EGFP⁺/EGFP⁺ individuals to putative EGFP⁺ heterozygotes was only 16 : 84 for the transgenic line derived from the G₁ female and 23 : 205 for the transgenic line derived from the G₁ male. However, we deliberately selected only those individuals that displayed a brilliant and unambiguous green fluorescence and so probably missed putative homozygotes that did not display this level of brilliant fluorescence. While genetic and molecular confirmation of the putative homozygosity of these individuals is required, the appearance of two levels of green fluorescence in the G₃ progeny offers preliminary evidence that, at least with the actin5C promoter, EGFP⁺ homozygotes may be discernible from EGFP⁺ heterozygotes.

Figure 2 (A–C) shows the phenotype of transgenic *Ae. aegypti* containing the act5C:EGFP gene. Expression of EGFP was seen in all life stages except for eggs that had undergone melanization. Expression of EGFP was seen during all four larval instars and was associated with the gut, the fat body tissue surrounding the gut, and in the gastric caeca (Fig. 2A). Distinct green fluorescence was observed in pupae (Fig. 2B). In adults green fluorescence was most obvious in the anterior region of the abdomen in both males and females (Fig. 2C). The presence of EGFP in the anterior abdomen is probably due to expression of EGFP in the midgut while, in females, the presence of EGFP

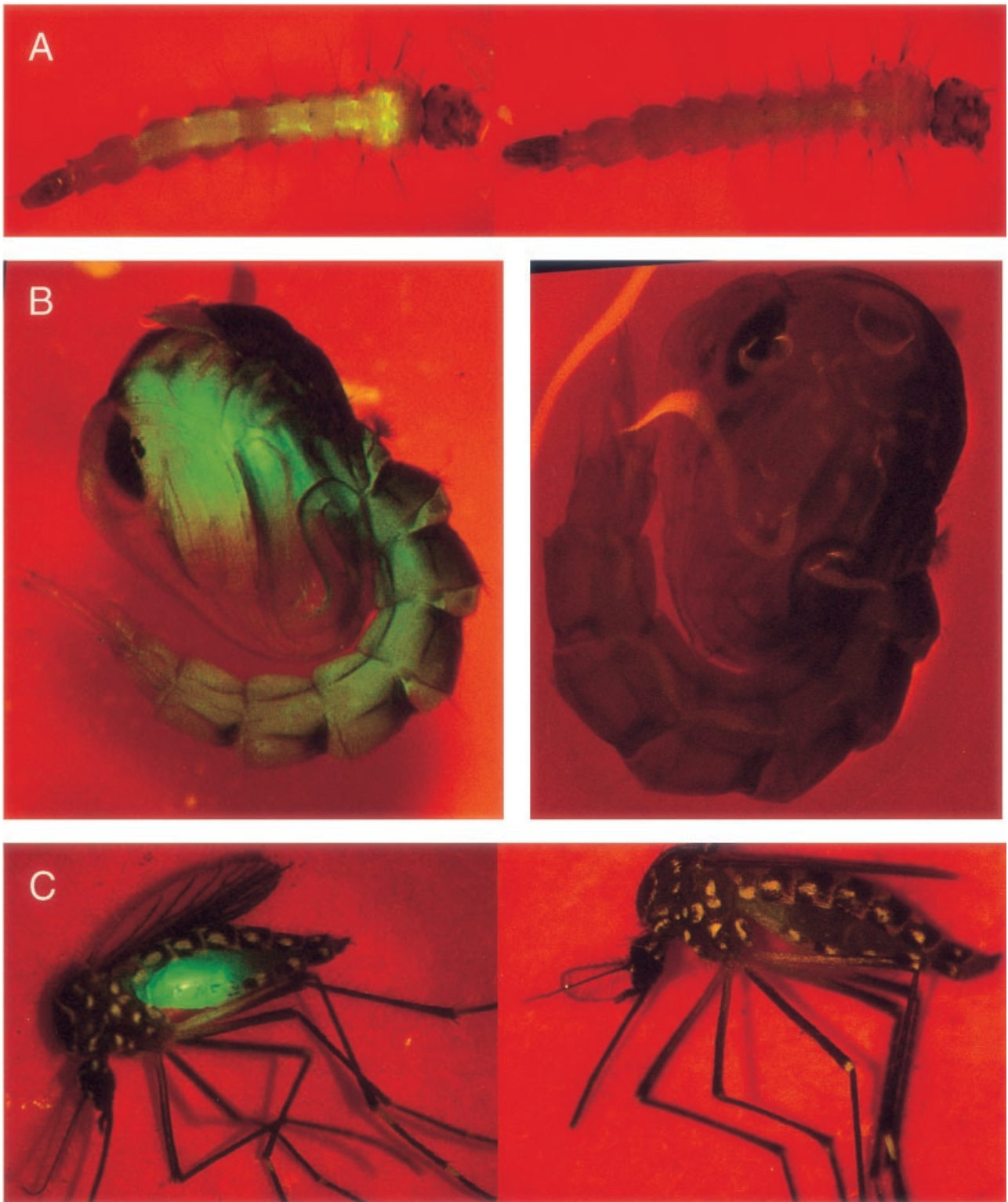


Figure 2. Expression of EGFP in transgenic *Ae. aegypti* containing the Hermes[act5C:EGFP] transposable element compared with non-transformed, wild-type mosquitoes. (A) A G_4 early instar larva that is intensely fluorescent and most likely homozygous for the Hermes[act5C:EGFP] element. Non-transformed early instar larva is also shown. (B) A G_4 pupa that is intensely fluorescent and most likely homozygous for the Hermes[act5C:EGFP] element. Non-transformed pupa is also shown. (C) A G_5 adult female 2–3 days following a blood meal showing intense fluorescence in the anterior section of the abdomen. She is most likely homozygous for the Hermes[act5C:EGFP] element. Non-transformed adult female is also shown.

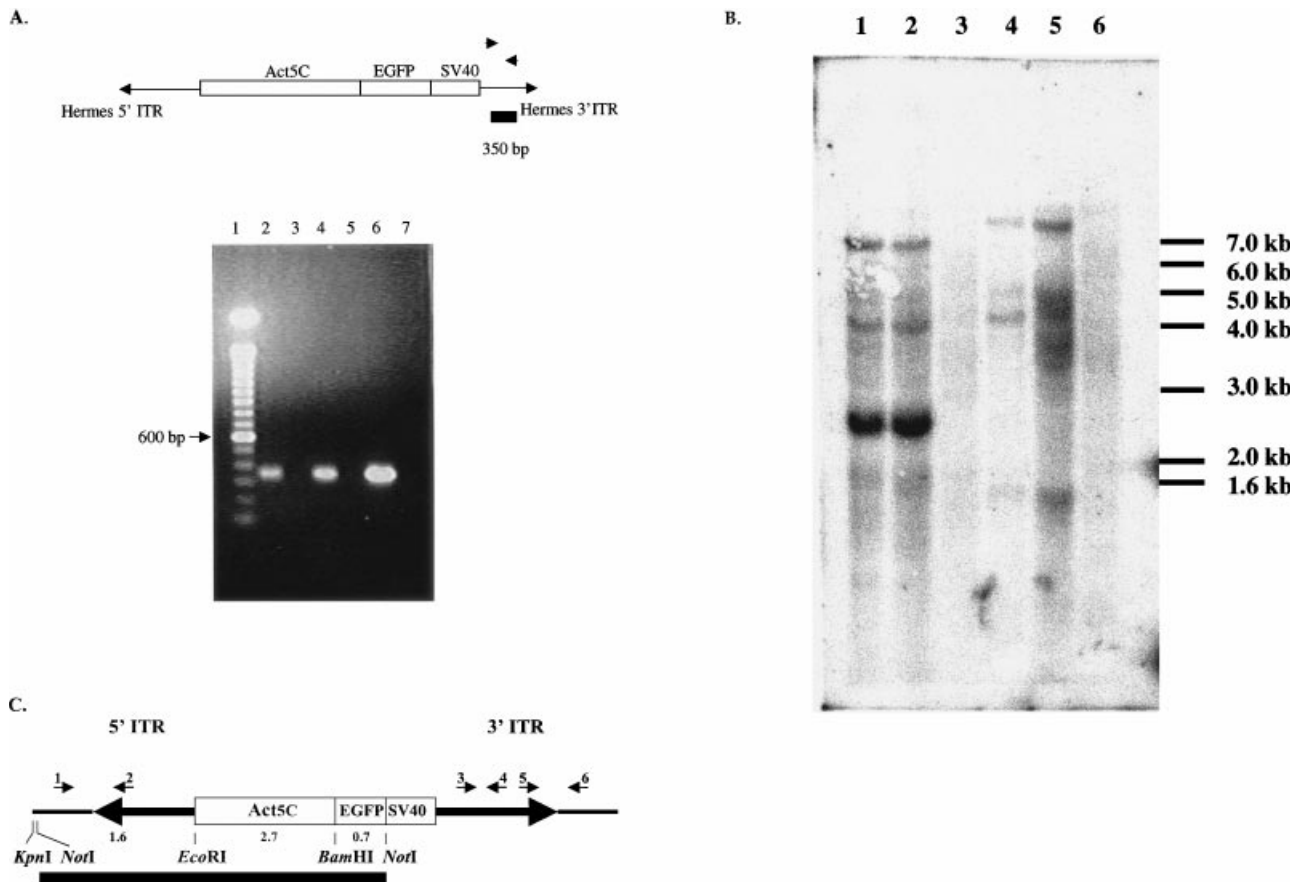


Figure 3. (A) Amplification of a 350 bp fragment using the 3 and 4 primers from the 3' end of the *Hermes* element in transgenic lines of *Ae. aegypti* (see diagram). Identical reactions with DNA prepared from non-transgenic siblings did not amplify this, or any other fragment. Lane 1: 100 bp molecular weight marker (Gibco-BRL). Lane 2: Genomic DNA from G₁ EGFP⁺ male. Lane 3: Genomic DNA from G₁ EGFP⁻ sib. Lane 4: Genomic DNA from G₁ EGFP⁺ female. Lane 5: Genomic DNA from G₁ EGFP⁻ sib. Lane 6: Plasmid pHermes[act5C:EGFP]. Lane 7: 0 DNA control. (B) Southern blot of genomic DNA prepared from transgenic G₂ and G₃ generations derived from the G₁ male line and non-transgenic *Ae. aegypti*. Genomic DNA was digested with the restriction enzymes shown and hybridized with a 5 kb *NotI* fragment that contained the 5' end of the *Hermes* element, the act5C:EGFP gene and approximately 900 bp of flanking *M. domestica* genomic DNA. Lane 1: Genomic DNA prepared from G₂ transgenic adults derived from the transgenic G₁ male. Digested with *BamHI* and *EcoRI*. Lane 2: Genomic DNA prepared from G₃ transgenic adults derived from the transgenic G₁ male. Digested with *BamHI* and *EcoRI*. Lane 3: Wild-type DNA digested with *BamHI* and *EcoRI*. Lane 4: Genomic DNA prepared from G₂ transgenic adults derived from the transgenic G₁ male. Digested with *KpnI* and *EcoRI*. Lane 5: Genomic DNA prepared from G₃ transgenic adults derived from the transgenic G₁ male. Digested with *KpnI* and *EcoRI*. Lane 6: Wild-type DNA digested with *KpnI* and *EcoRI*. The relative positions of the molecular weight marker fragments are shown. (C) Diagram showing the map of the Hermes[act5C:EGFP] element and flanking *M. domestica* genomic DNA. Distances shown are in kilobases. The bar underneath the map shows the 5 kb *NotI* fragment used as the probe in the Southern hybridizations. Numbered arrows above the map show the locations of the oligonucleotide primers used in the PCR analysis of transgenic lines.

more posterior in the abdomen was correlated with the development of the ovaries. Expression of EGFP was particularly clear in gravid females. In all stages, the expression of the act5C:EGFP gene was consistent with what is predicted from a marker gene placed under the control of a cytoskeletal actin promoter (Burn *et al.*, 1989). Similar tissue and developmental patterns of EGFP expression were observed in transgenic *D. melanogaster* lines containing the same Hermes[act5C:EGFP] element.

Molecular analysis of the transgenic lines confirmed that the Hermes[act5C:EGFP] sequences were present in the individuals that displayed green fluorescence and were absent from their sibs that did not fluoresce. Initially we

used PCR analysis to confirm the presence of *Hermes* sequences in the transgenic lines. Primers 3 and 4 from the *Hermes* 3' end were used to amplify the expected 350 bp fragment from both transgenic lines (Fig. 3A, C). No fragment of any size was observed in genomic DNA prepared from non-fluorescing sibs (Fig. 3A). Southern blots also confirmed the presence of the Hermes[act5C:EGFP] element (Fig. 3B). Transgenic and non-transgenic DNA was double digested with *EcoRI*-*BamHI* and *EcoRI*-*KpnI*, fractionated, blotted and hybridized against a 5 kb *NotI* fragment that consisted of the 5' end of the *Hermes* element, the actin5C promoter sequences, the EGFP gene and approximately 900 bp of *Musca domestica* DNA flanking the 5'

end of the *Hermes* element. As shown in Fig. 3(B), all Hermes[act5C:EGFP] sequences were present in the transgenic lines. The 2.7 kb *EcoRI*-*Bam*HI fragment containing the actin5C promoter was clearly visible as were two extra fragments approximately 4 kb and 7 kb in size. These hybridizing fragments extend into the *Ae. aegypti* genome since there are no other *EcoRI* or *Bam*HI sites in the pHermes[act5C:EGFP]. We conclude that the breakpoints of integration are located within 4–7 kb either side of the 2.7 kb *EcoRI*-*Bam*HI fragment containing the actin5C promoter. No hybridizing fragments were seen in genomic DNA prepared from wild-type *Ae. aegypti*. Analysis of hybridizing fragments resulting from a *KpnI*-*EcoRI* double digest of genomic DNA prepared from transgenic and non-transgenic *Ae. aegypti* indicates that two fragments of approximately 7.5 kb and 4.2 kb hybridized to the 5.0 kb *NotI* probe. Interestingly a 1.6 kb *KpnI*-*EcoRI* fragment that would result from integration of the *Hermes* element together with all of the *M. domestica* genomic DNA flanking the 5' end of this element was absent. This indicates that the 5' breakpoint is located before the *KpnI* site in the *M. domestica* flanking DNA which is approximately 890 bp upstream from the first nucleotide of *Hermes*.

To further locate the junction fragments of the Hermes[act5C:EGFP] gene, an IPCR approach was undertaken. Primers specific for the terminal regions of the *Hermes* element and, in some cases, flanking *M. domestica* DNA, were used (Fig. 3C). This analysis revealed that flanking *M. domestica* DNA had integrated into the genome of *Ae. aegypti* along with the Hermes[act5C:EGFP] element. This was subsequently confirmed by PCR using primers that amplify regions across the *Hermes* ITRs into flanking *M. domestica* DNA and also by using primer pairs specific only for the *M. domestica* flanking DNA. Primer 1 (Fig. 3C) located approximately 640 bp upstream from the *Hermes* 5' ITR in *M. domestica* genomic DNA flanking the 5' end of the *Hermes* element and primer 2 from the 5' end of *Hermes* amplified a fragment 730 bp in size from genomic DNA prepared from transgenic mosquitoes but not their non-transformed sibs (data not shown). Similarly, primer 6 located approximately 60 bp downstream from the *Hermes* 3' ITR in *M. domestica* genomic DNA flanking the 3' end of the *Hermes* element and primer 5 from the 3' end of the *Hermes* element amplified a fragment 300 bp in size from genomic DNA prepared from transgenic mosquitoes but not their non-transformed sibs (data not shown). While the exact breakpoints are yet to be located, we conclude that the entire Hermes[act5C:EGFP] element has integrated, as an intact sequence, into the *Ae. aegypti* genome together with flanking *M. domestica* genomic DNA. The 5' breakpoint of integration is located between approximately 640 bp and 890 bp upstream of the *Hermes* 5' ITR while the location of the 3' breakpoint is more than 60 bp downstream from the *Hermes* 3' ITR.

Discussion

We have generated transgenic lines of *D. melanogaster* and *Ae. aegypti* using the EGFP gene as the primary genetic marker. These results demonstrate that this gene can be used as a genetic marker in at least these species. In particular the ability to identify genetic transformants of a wild-type strain of mosquito indicates quite strongly that the EGFP gene will be a useful genetic marker in this, and other, mosquito species such as *Anopheles gambiae* and *Culex* species.

Transformation of *D. melanogaster* was mediated by transpositional recombination of the *Hermes* element. The frequency of transformation was consistent with previous examples of *Hermes*-mediated transformation of this species. Furthermore all the hallmarks of *Hermes*-mediated transposition were observed in these transgenic lines. The sequences integrated were delimited by the terminal nucleotides of the *Hermes* ITRs and an 8 bp duplication was created at the target site. The sequence of this target site duplication conformed to the consensus target site sequence observed for integrations of the *Hermes* element and other *hAT* elements into plasmids and insect chromosomes (O'Brochta *et al.*, 1994, 1996; Pinkerton *et al.*, 1996; Sarkar *et al.*, 1997a,b).

Expression of EGFP in these transgenic *D. melanogaster* lines was consistent with the expression of a reporter gene under the control of the actin5C promoter. The actin5C gene encodes a cytoskeletal actin which is found in the microfilaments of all cells but is also present in higher amounts in specific tissues (Burn *et al.*, 1989). The expression of this gene is particularly noticeable in cells that are undergoing rapid cell division such as those comprising the gonadal tissue of both sexes. It is also highly expressed in the cells of the embryonic midgut and proventriculus (Burn *et al.*, 1989). In some transgenic lines expression of EGFP was seen throughout the insect; in other lines it was observed most strongly in cells comprising the embryonic gut and in cells comprising the ovarioles of adult females. While we did not directly measure either the fitness or fecundity of these transgenic lines, from routine handling of these cultures, there was no obvious difference in the viability of these EGFP-expressing transgenic lines relative to the non-transformed flies.

The same vector plasmid used to transform *D. melanogaster* was also used to genetically transform *Ae. aegypti*. The transformation frequency obtained, 2.3–3.8%, is consistent with that obtained by others who have used the *Hermes* element to mediate the genetic transformation of *Ae. aegypti* (Jasinskiene *et al.*, 1998). While two separate transformed G₁ individuals, a male and female, were obtained, both originated from the same pool mating and most likely were derived from the same original integration event in the developing gametes of a single G₀ female.

This was also suggested by Southern blot and IPCR analysis that showed no molecular differences between the two transgenic lines.

The pattern of expression of the EGFP gene in successive generations was Mendelian in nature and was consistent with the integration of the EGFP gene at a single location in the *Ae. aegypti* genome. Based on the inheritance of this gene over four generations, the integration of the EGFP gene appeared stable. Expression of the EGFP gene was observed through all life stages of the mosquito with the exception of eggs that had undergone melanization after which the opaqueness of the chorion prevented visualization of the developing embryo. In larvae, expression of EGFP was observed principally in the fat body, the gut and in the gastric caeca. It was also detectable in the head following removal of the cuticle. In pupae, expression was throughout the individual while in adults expression of the EGFP gene was most noticeable in gonadal tissue. The pattern of larval expression of the EGFP gene was different to what was seen in *D. melanogaster* transformants. Restriction of the strongest expression of this gene to the fat body is probably due to the polyploidy of this tissue. Once again, while direct measurements of fecundity and viability of the transgenic lines relative to non-transformed strains have not, as yet, been made, the expression of EGFP in these lines appears to have no demonstrable effect on their survival. However, this is one subject we propose to examine more closely in both heterozygous and homozygous lines.

Most significant was the fact that the EGFP gene was successfully used to identify genetic transformants of wild-type mosquitoes. Transgenic *Ae. aegypti* larvae were easily recognized from their non-transformed sibs using a dissecting microscope and appropriate excitation and detection conditions. The initial identification of the two transgenic G₁ larvae was made when both were surrounded by approximately 250 of their non-transformed sibs. Identification was clear, quick and unequivocal. This ease of transgenic identification was confirmed when subsequent generations of these transgenic lines were examined and transgenic heterozygotes needed to be identified and sorted from their non-transgenic sibs. In addition, as mentioned above, transgenic larvae and adults homozygous for the act5C:EGFP transgene can be identified from transgenic heterozygotes.

Unlike *Hermes*-mediated transformation of *D. melanogaster*, integration of the *Hermes* element in *Ae. aegypti* does not bear the hallmarks of normal transpositional recombination. In addition to the sequences bound by the ITRs of the *Hermes* element, *M. domestica* genomic DNA flanking the element has also integrated into the mosquito genome. Both 5' and 3' breakpoints are located in *M. domestica* genomic DNA flanking the *Hermes* element although their precise location is yet to be determined.

At this stage it is not possible to determine whether or not integration of the Hermes[act5C:EGFP] element and flanking sequences has occurred through a recombination mechanism that is dependent or independent of the *Hermes* transposase. Interplasmid transposition assays performed in *Ae. aegypti* have demonstrated that the *Hermes* element can accurately transpose in this mosquito (Sarkar *et al.*, 1997b). Furthermore the *Hermes* element can accurately transpose into the genome of another mosquito species, *Anopheles gambiae* (Zhao & Eggleston, 1998). In this case an established cell line of *An. gambiae* was used and three independent precise *Hermes* transpositions into the mosquito genome were recovered, each of which bore all the hallmarks associated with *hAT* element transposition.

The type of *Hermes* integration event observed here is very similar in structure to those characterized from three transgenic lines of *Ae. aegypti* in which the *D. melanogaster cinnabar* gene was used as the genetic marker (Jasinskiene *et al.*, 1998; Coates *et al.*, 1999; Jasinskiene *et al.*, in press). In these, *M. domestica* flanking sequences had also integrated, along with the *Hermes* element, into the *Ae. aegypti* genome. Failure to achieve transgenic lines of *Ae. aegypti* when these experiments were repeated, but in the absence of helper plasmid, suggest that these types of *Hermes* integration events do, in fact, require the presence of the *Hermes* transposase (Jasinskiene *et al.*, 2000).

The difference in the molecular basis of transformation between *D. melanogaster* and *Ae. aegypti* is striking. The *D. melanogaster* strain used is devoid of *hobo* elements while it is not known whether *hAT*-like elements are present in *Ae. aegypti* although there is some evidence that suggests that they may (Jasinskiene *et al.*, in press). Whether related transposable elements or host-related factors are responsible for the differences in the integration events recovered from these two insect species remains to be examined.

We have shown that the EGFP gene can be easily and effectively used as the primary genetic marker to identify transgenic individuals in two species of insects, *D. melanogaster* and *Ae. aegypti*. In *Ae. aegypti* the use of this marker will greatly expand our ability to introduce genes into this species since wild-type strains of *Ae. aegypti* can now be used as recipients. Furthermore the successful demonstration of the use of EGFP as a marker in *Ae. aegypti* bodes well for its use in other mosquito species such as *An. gambiae* and *Culex* species. There are, however, many questions regarding the expression of the EGFP in the extant lines of *Ae. aegypti*. These questions concern the possible effects that EGFP expression may have on fertility, fecundity, longevity and viability. The spread of a transposable element containing the EGFP gene through caged mosquito populations can now be examined once related questions regarding the stability

and remobilization of the *Hermes* element containing the act5C:EGFP gene have been addressed. Examination of these questions will be the subject of future communications.

Experimental procedures

Mosquitoes

A wild-type strain of *Ae. aegypti* 'Orlando' was used as the recipient strain. This wild-type strain has been reared in the Department of Entomology at the University of California for several years under standard conditions (Munstermann, 1997) with the exception that larvae were fed on a modified diet rather than a fish food diet in order to reduce background fluorescence associated with fish food. The modified larval diet consisted of ground Milkbone Original Dog Biscuits mixed with 'Red Star' Specialty Nutrex 55 in a 2 : 1 ratio.

Plasmid constructions

The plasmids pKhs82Hermes and pSHH14 have previously been described (Sarkar *et al.*, 1997a). Plasmid pHermes[act5C:EGFP] was constructed by cloning the 2.7 kb *EcoRI*-*Bam*HI fragment from pCaSpeR-act (Thummel *et al.*, 1988) containing the *D. melanogaster* actin5C promoter into the *EcoRI*-*Bam*HI sites in plasmid pHermes[EGFP]. Plasmid pHermes[EGFP] was constructed by cutting plasmid pEGFP-1 (Clontech) containing the EGFP gene and the SV40 polyadenylation signal with *Afl*III and then blunt ending this site with Klenow polymerase. This linearized plasmid was then cut with *Eco*RI producing a 950 bp *Afl*III-*Eco*RI fragment which was gel purified and cloned into the plasmid pBSHermesBam. This plasmid had been initially cut with *Hind*III after which this *Hind*III site was blunt-ended using Klenow polymerase. This *Hind*III cut linearized plasmid was then cut with *Eco*RI and the ligated with the 950 bp EGFP-SV40 fragment. Plasmid pBSHermesBam was constructed by cutting pBSHermes (O'Brochta *et al.*, 1996) with *Bam*HI, blunt ending with Klenow polymerase and re-ligating to produce a plasmid that contained no *Bam*HI sites.

Genomic DNA preparation

Mosquito genomic DNA was prepared according to the technique of Jasinskiene *et al.* (in press).

Gene amplification

The following *Hermes*-specific primers were used to confirm the presence of *Hermes* in transgenic mosquitoes (see Fig. 3C):

Primer 3: 5'-GACTGGAAAAAAT TTAAGTTTAAAAGAAGC-3'

Primer 4: 5'-CACATAGATAAGCACAAAGTGT TTTGG-3'.

The following *Hermes*-specific primers and *M. domestica* flanking DNA specific primers were used to identify the approximate locations of the breakpoints in transgenic mosquitoes (see Fig. 3C):

Primer 1: 5'-TTGCT TATTTTCAGT TACCAGCAAAC-3'

Primer 2: 5'-AATGAATTT TTTGT TCAAGTGGCAAAGCAC-3'

Primer 5: 5'-AATTTGCCAAT TGACAAATCGCACACGTCC-3'

Primer 6: 5'-ATCCGACCAT TCATTGGCTAT TTTCCAATC-3'

Reaction conditions were performed as follows: 200 ng of genomic DNA prepared from EGFP⁺ or EGFP⁻ adults (with heads removed) was added to a solution containing Fisherbrand Taq polymerase (1.5 U) and Fisherbrand Taq buffer and 20 pmol of each primer

and the final reaction volume taken to 30 μ l. Thin-walled tubes 0.2 ml (MJ Research) containing the reaction mix were placed in an MJ PTC 200 DNA engine. Thermocycling conditions were 94 °C, 4 min followed by forty cycles at 94 °C, 30 s; 60 °C, 15 s; and 72 °C, 1 min. A final extension step at 72 °C, 4 min then followed. Reaction products were then analysed by gel electrophoresis using 1–1.5% agarose gels depending on fragment size.

Insect embryo microinjections

Drosophila melanogaster embryo microinjections were performed according to standard procedures (Rubin & Spradling, 1982). *Aedes aegypti* embryo microinjections were performed according to standard techniques for this species (Morris, 1997).

Southern hybridizations

Southern blot analysis was performed as described (Sambrook *et al.*, 1989). The *Hermes* probe used was a 5.0 kb fragment resulting from a *Not*I digest of plasmid pHermes[act5C:EGFP]. It contained approximately 900 bp of 5' flanking *M. domestica* DNA, approximately 700 bp of the *Hermes* element 5' end, 2.7 kb of the *D. melanogaster* actin5C promoter and approximately 700 bp of the structural region of the EGFP gene. This *Not*I fragment was gel purified and radiolabelled with ³²P α dATP using random primer labelling (Stratagene). Transfer on to Qiabrane Nylon Plus membrane (Qiagen Inc.) was achieved using a Posiblot apparatus and pressure control station (Stratagene) and cross-linking under ultraviolet light. Hybridizations and washings were performed under stringent conditions. Hybridization buffer was 0.3 M sodium phosphate buffer (pH 7.2), 7% sodium dodecyl sulphate (SDS) and hybridizations were performed at 65 °C. Washes were performed twice in 2 \times SSC, 0.1% SDS at 65 °C and then twice in 0.1 \times SSC, 0.1% SDS at 65 °C. Filters were placed in a Phosphor Screen (Molecular Dynamics) and images obtained using a molecular dynamics phosphorimager.

Enhanced green fluorescent protein detection

Adult insects were immobilized with carbon dioxide while larvae and pupae were immobilized by placing the insects into a prechilled Syracuse watch glass which was placed on a chilled stage during microscopy. Insects containing EGFP were detected using a Leica MZ12 stereomicroscope to which was attached a Leica fluorescence module. A GFP Plus fluorescence filter set consisting on an excitation filter (480/40 nm) and a barrier filter (510 nm) were used.

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