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Transformation of *Stomoxys calcitrans* with a *Hermes* gene vector

D. A. O'Brochta,[1] Peter W. Atkinson[2] and Michael J. Lehane[3]

[1] *Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, MD,* [2] *Department of Entomology, University of California, Riverside, CA, USA,* [3] *School of Biological Sciences, University of Wales, Bangor, Gwynedd, UK*

Abstract

The ability of the *Hermes* transposable element to function as a germ line transformation vector was tested in the stable fly, *Stomoxys calcitrans*. Plasmid-based transposable element mobility assays indicated moderate mobility of *Hermes* in this species. Germline transformants were created using a *Hermes* element containing the enhanced green fluorescent protein (EGFP) under the regulatory control of the promoter from Actin5C gene of *Drosophila melanogaster*. Approximately 4% of the fifty-five adults that developed from the 1903 G_0 embryos injected with the vector produced transgenic progeny. In the four transgenic lines established, the EGFP expression pattern was distinctly nonuniform and levels of expression were low. Promoters other than the one from the Actin5C gene of *D. melanogaster* should be considered for widespread, constitutive expression. All transgenic lines contained multiple (2–4) integrated *Hermes* elements. *Hermes* integration events occurred through a canonical cut-and-paste mechanism.

Introduction

The biting fly *Stomoxys calcitrans* (stable fly, family Muscidae) is widely distributed in the New and Old World and is an economically important pest of cattle. Stable flies can also act as mechanical vectors of trypanosomes but are not considered important vectors of this disease (Lehane, 1991). Bruce & Decker (1958) reported a significant negative correlation between *S. calcitrans* numbers and milk production in dairy cows kept in fields. Furthermore, the effects of *S. calcitrans* on milk production are exacerbated when food is limited (Miller *et al.*, 1973). Consequently, *S. calcitrans* can be a significant problem in feedlots when densities exceed a certain threshold because significantly more food must be supplied to achieve the productivity levels seen in *S. calcitrans*-free animals (Campbell *et al.*, 1987; Cantangui *et al.*, 1995). Economic infestation thresholds in feedlots can be as low as seven stable flies per foreleg per animal per minute (Cantangui *et al.*, 1997). The total losses to the North American cattle industry caused by stable flies were estimated in 1987 at US$ 428 million per year (Drummond, 1987).

The haematophagous habits of adult flies, their size and the ease with which they can be bred in the laboratory have made *S. calcitrans* a valuable model insect for investigating midgut physiology of blood sucking insects. (e.g. Moffatt *et al.*, 1995; Lehane & Lehane, 1997). The midgut of blood sucking insects is a crucial interface between vectors and parasites that are ingested during feeding. Until recently, little was known about the midgut immune system in insects and its role in insect–parasite interactions despite the practical importance to man of insect vectors of disease. It is now known that the midguts of *S. calcitrans* and the mosquito *Anopheles gambiae* secrete defensins, a class of antimicrobial peptide (Dimopoulos *et al.*, 1998; Lehane & Lehane, 1997). Despite the demonstrated role of the midgut in the innate immune response, we lack suitable tools for gaining a full understanding of the role the midgut plays as the interface between vectors and parasites such as *Plasmodium, Leishmania, Typanosoma*, *Wuchereria*, *Yersinia* and arboviruses. The recent development of gene transformation systems for nondrosophilid insects promises to provide new opportunities to isolate insect immune-related genes and to understand their function and regulation. Here we demonstrate that the insect gene vector based on the *Hermes* transposable element is functional in *S. calcitrans* and can be used to create transgenic lines of this species.

Hermes is a short inverted repeat-type transposable element 2.7 kb in length containing a 618 amino acids open reading frame encoding an essential transposase protein, and 17 bp terminal inverted repeat sequences

Received 14 April 2000; accepted following revision 10 July 2000. Correspondence: David A. O'Brochta, Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, MD 20742–4450, USA. E-mail: obrochta@umbi.umd.edu

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essential for transposition and excision (Atkinson *et al.*, 1993; Warren *et al.*, 1994). *Hermes* is a member of the *hAT* (*hobo*, *Ac*, *Tam3*) family of transposable elements showing particularly clear affinities to the *hobo* element of *D. melanogaster*. The transposase proteins of the *Hermes* and *hobo* elements are 55% identical and 70% similar at the amino acid level (Atkinson *et al.*, 1993; Warren *et al.*, 1994). The terminal inverted repeats of these elements are also very similar with ten of the twelve terminal nucleotides of the left end and eleven of twelve nucleotides of the right end of *hobo* identical to the corresponding bases of the *Hermes* element, further reflecting the close relationship between these transposons (Warren *et al.*, 1994). *hAT* elements in general, and *Hermes* specifically, have broad host ranges. For example, *Hermes* mobility has been observed in at least twelve species of insects (Atkinson & O'Brochta, 1999 and unpublished data) and germ line integration of *Hermes* leading to the creation of transgenic insects has been accomplished in four species of Diptera, *D. melanogaster* (O'Brochta *et al.*, 1996), *Aedes aegypti* (Jasinskiene *et al.*, 1998), *Culex quinquefasciatus* (M. L. Allen, C. Levesque, D. A. O'Brochta and P. W. Atkinson, manuscript in preparation), *Ceratitis capitata* (Michel *et al.* in preparation) and a beetle, *Tribolium castenaeum* (Berghammer *et al.*, 1999b). The results reported here contribute to a growing body of data demonstrating the general utility of the *Hermes* transposable element system as an insect gene vector and provide the opportunity to investigate in greater detail the molecular genetics of insect midgut immunity in an economically important pest of livestock.

Results

Plasmid-based mobility assays

We performed *Hermes* transposition assays at two independent locations (The University of Wales and The University of Maryland Biotechnology Institute). Three independent experiments were performed at each location. *Hermes* transpositions were readily detected in *S. calcitrans* embryos with approximately three (2.79 ± 2.69) events occurring for every 10 000 donor elements injected and recovered (Table 1). The variance in the observed transposition rate was high and rates ranged from less than one to almost eight events per 10 000 donor elements recovered. The primary means by which putative recombinant plasmids were analysed was by restriction endonuclease mapping. All recombinant plasmids approximately 8.6 kb in length with two *Sal*I sites were scored as transposition events. A sample of the plasmids scored as transposition events were analysed by DNA sequencing to confirm the validity of our scoring criteria. The sequence of recombinant plasmids arising from *Hermes* transposition into the target plasmid suggested that only sequences precisely delimited by the terminal inverted repeats of *Hermes* were involved in the transposition reaction and all resulted in an 8 bp direct duplication of the target sequence (Fig. 1). Directly transforming *E. coli* with the mixture of plasmids being injected into embryos failed to recover any recombinant

Table 1. Plasmid-based *Hermes* transposition assay

Experiment	Replicate	Donors	Transpositions	Rate per 10^4
I	1	22 240	2	0.8993
	2	176 500	140	7.9320
	3	160 000	8	0.5000
			Mean	3.11
II	4	30 000	7	2.3333
	5	39 000	12	3.0769
	6	20 000	4	2.0000
			Mean	2.47

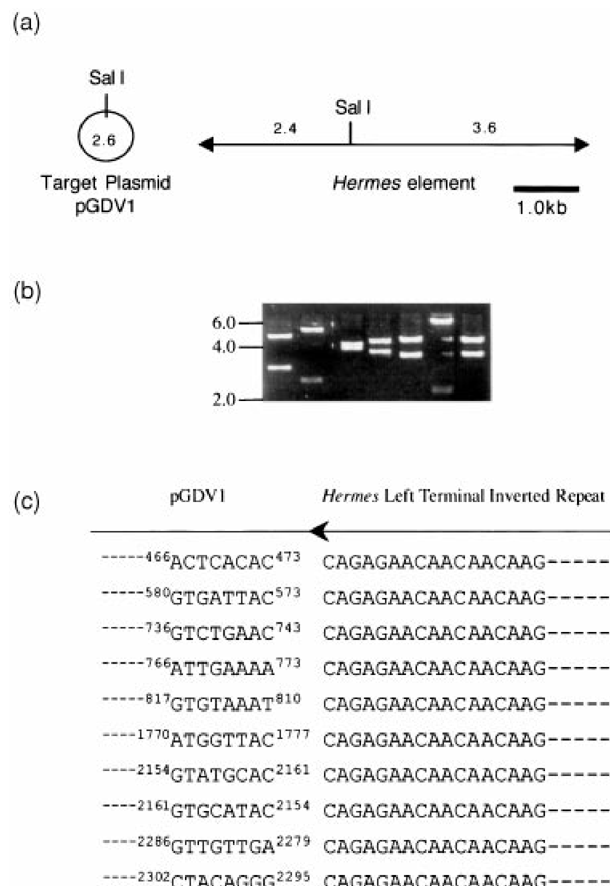

Figure 1. Plasmid-based *Hermes* mobility assay. (a) Diagrammatic representation of the 2.6 kb target plasmid, pGDV1, and the *Hermes* element contained within the donor plasmid. The positions of the *Sal*I restriction sites are shown. (b) Seven recombinant plasmids that meet the physical criteria used to score a transposition event. Each plasmid is approximately 8.5 kb and has two *Sal*I sites. (c) DNA sequence of *Hermes* integration sites within pGDV1. Only the junction between the left terminal inverted repeat of *Hermes* and the target plasmid is shown along with the eight flanking nucleotides forming a direct repeat. Numbers associated with nucleotides refer to positions within pGDV1 and are consistent with those reported by Sarkar *et al.*, 1997a,b.

plasmids meeting our criteria for being scored as a transposition event ($n > 10^6$ donor plasmids screened). All transposition events recovered arose post injection.

Germline transformation

A total of 1903 eggs were injected with the vector pHermes(A5CEGFP) and the helper plasmid pHSHH1.9. Fifty-five per cent (1075) of the injected eggs developed and subsequently hatched and 365 survived larval development and pupated. However, only fifty-five of the recovered pupae hatched. From these fifty-five G_0 adults two (#23 and #40) produced EGFP offspring, which subsequent analysis confirmed were transgenic (see below). The observed germ line transformation efficiency was therefore approximately 4%. Four transgenic lines were established. Two lines were established from two EGFP G_1 males arising from G_0 progeny #23 (lines 23m1 and 23m2) and lines were established from one EGFP G_1 male and one EGFP G_1 female progeny arising from G_0 progeny #40 (lines 40m3, 40f18).

In all lines the EGFP expression levels were low and distinctly nonuniform with visible expression confined to relatively few cells of undetermined type. EGFP expression could not be easily seen through the cuticle of third-instar larvae, pupae or adults. The cuticle at these later stages is sufficiently opaque to impair the visibility of the low levels of EGFP expression associated with these lines. Dissections of transgenic larvae indicated that the cuticle is not quenching EGFP flourescence. The visible pattern of expression in intact young larvae accurately reflects the pattern of expression of EGFP. The most distinct pattern of expression was seen in lines 23m1 and 23m2 (Fig. 2). In these lines the pharynx and two symmetrically located clumps of cells (perhaps imaginal discs) located just posterior to the pharynx were consistently green. Line 40m3 had very limited expression just posterior to the pharynx in symmetrically positioned structures that appeared to be part of the tracheal system. Line 40f18 showed a pattern of expression that resulted in the larvae having a green stippled appearance with a heavier concentration of stippling occurring in abdominal segment 8. Although the patterns were subtle and difficult to record they were clear enough to ensure the successful selection and propagation of the lines over eleven generations (at the time of writing).

Molecular analysis of the integrated Hermes*(*A5CEGFP*)*

Southern blot analysis was performed using genomic DNA isolated from pools of adults collected from the third and eighth generations. Genomic DNA was digested with appropriate restriction enzymes and probed with a 500 bp probe specific for the right end of *Hermes* such that each integrated element should yield only a single band of hybridization. The number of integrated elements was estimated by counting the bands of hybridization (Fig. 3). In the third generation lines 23m1 and 23m2 appeared to contained approximately two integrated elements while lines 40m3 and 40f18 appeared to contain approximately four and three elements, respectively (Fig. 3). Lines 23m1 and 23m2 had patterns of hybridization that were indistinguishable, consistent with them originating from G_0 male #23. Lines 40m3 and 40f18 had similar but distinct patterns of hybridization. At G_3 40m3 had clear evidence of at least four elements while 40f18 appeared to have only three elements. Two of the three elements in 40f18 were also present in 40m3. After five additional generations the pattern of hybridization seen in 23m1 and 23m2 was identical to that observed at G_3 (Fig. 3). Lines 40m3 and 40f18 appeared to have one less *Hermes* element at G_8 compared to G_3. Under the hybridization conditions used, wild-type *S. calcitrans* displayed no evidence of *Hermes*-hybridizing sequences.

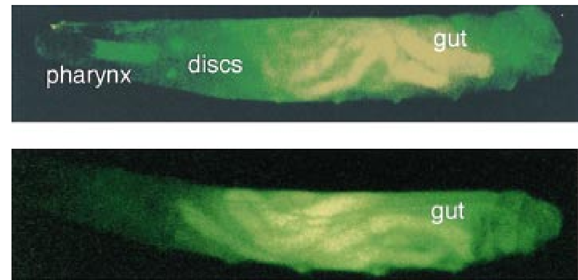

Figure 2. EGFP expression pattern in line 23m1. Shown are second-instar larvae viewed with a Leica MZFLIII fluorescence stereomicroscope equipped with a GFP2 filter. A transgenic larva is shown above and a wild-type larva is shown below. Both images were intentionally overexposed to enhance the visibility of the green pharynx and two groups of green cells located just posterior to the pharynx, which might belong to developing imaginal discs in the transgenic individual. The diffuse green glow located throughout the rest of the image is not readily discernible when the larva was viewed directly through the microscope. The yellow convoluted structure located medially is the gut. The yellow fluorescence results from auto-fluorescence of the gut contents and is not the result of EGFP expression.

To investigate the nature of the integration events we cloned fragments of DNA that included the terminal inverted repeats of some of the integrated *Hermes* elements and the DNA immediately flanking the terminal sequences using inverse PCR methods. We obtained DNA sequence information for five integrated elements. In no case were sequences originally flanking the termini of *Hermes* in the input plasmid found flanking the integrated element. In two cases we isolated the right and left ends of the integrated element and in both cases an 8 bp direct duplication of the integration site was found (Fig. 4). The 8 bp sequences flanking the integrated *Hermes* elements were not distinctly different from those found flanking *Hermes* elements integrated into the genome of other insects and target plasmids (O'Brochta & Atkinson, 1996; Sarkar *et al.*, 1997a, b).

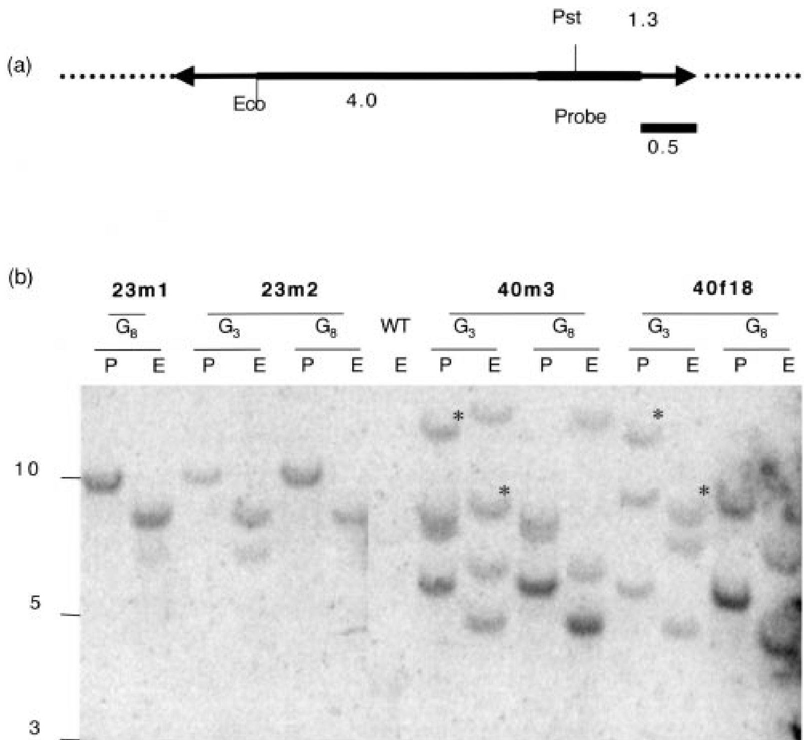

Figure 3. Southern blot analysis of *S. calcitrans* containing integrated *Hermes* gene vectors. (a) Diagrammatic representation of an integrated *Hermes* element showing the positions of the *Pst*I (P) and *Eco*RI (E) sites within the element and their distance in kilobases from the right terminal inverted repeat. The location and the size (in kb) of the sequence used as a probe is also indicated. (b) Results of a Southern blot analysis of four transgenic lines (23m1, 23m2, 40m3, 40f18) at generations 3 (G_3) and 8 (G_8). Genomic DNA was digested with *Pst*I (P) and *Eco*RI (E). The positions of the 3, 5 and 10 kb molecular markers are shown. The asterisks identify bands that were present only at G_3 Data from line 23m1 at G_3 is not shown.

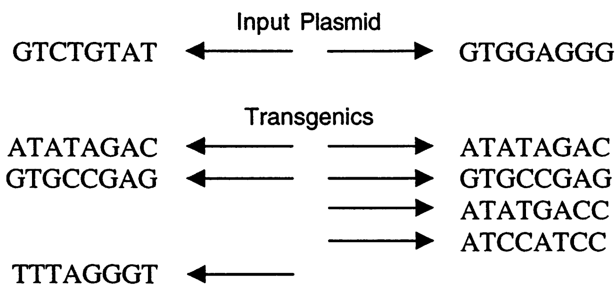

Figure 4. Analysis of integrated *Hermes* elements. DNA flanking the integrated element was cloned using an inverse PCR strategy. Shown are the left and right inverted terminal repeats of *Hermes* (left and right facing arrow, respectively) and the first eight nucleotides of flanking DNA. As a comparison the sequences flanking the *Hermes* element on the donor plasmid are shown. Note the vector used was constructed from right and left terminal inverted repeats from independently isolated *Hermes* elements from *M. domestica* and therefore do not have the same eight nucleotides of flanking DNA (Warren *et al.*, 1994; O'Brochta & Atkinson, 1996).

Discussion

The data reported here demonstrate the mobility of *Hermes* in the stable fly, *S. calcitrans*, and add to our understanding of the mobility potential and properties of this element in insects. Plasmid-based transposable element mobility assays have been used regularly as a preliminary indicator of a transposable element's mobility properties in a species of interest (O'Brochta & Handler, 1988; Sarkar *et al.*, 1997a,b; Thibault *et al.*, 1999). To date, the results of these assays have been good indicators of an element's ability to serve as a germ line transformation vector in the species being tested. Transpositional recombination rates calculated from plasmid-based assays indicated that *Hermes* was moderately active in *S. calcitrans* relative to the rates observed in other species. For example, Sarkar *et al.* (1997a) reported rates of *Hermes* transposition in *M. domestica* ranging from 0.5 to 1.4 events per 10 000 donor plasmids screened while in *S. calcitrans* the rate was two to five times higher. Observed rates in *D. melanogaster* and *C. capitata* were as high as fifteen events per 10 000 donor plasmids screened (Sarkar *et al.*, 1997a). The data reported here are consistent with earlier findings that *Hermes* is an active element in cyclorrhaphan flies. Furthermore, the characteristics of the integration events recovered in *S. calcitrans* are similar to those described from other insects. A typical insertion site duplication of 8 bp was created during the integration reaction and while there is no strong consensus sequence that defines a *Hermes* integration site the integration sites that were examined did not differ greatly from those described in other species (K. Michel *et al.* in preparation; O'Brochta & Atkinson, 1996; Sarkar *et al.*, 1997a,b). In conclusion the properties of the *Hermes* element with respect to recombination rates and target site selection seem to be consistent within the Diptera.

Short inverted repeat type transposable elements commonly move by employing a cut-and-paste type mechanism (Berg & Howe, 1989). This mechanism involves the excision and transposition of only sequences precisely delimited by the terminal inverted repeats of the element. Non-canonical transposition events in which sequences flanking an element are also involved in the transposition reaction have been reported. For example, the description of some of the first *P* element transformants of *D. melanogaster* by Rubin & Spradling (1983) included at least one non-canonical event. Subsequently other noncanonical *P* element transposition events have been described (Tsubota & Dang-Vu, 1991; Gray *et al*., 1996). Jasinskiene *et al*. (2000) described *Aedes aegypti* transformed with a *Hermes* vector and based on their analysis integration appears to have occurred by a noncanonical transposition reaction. The germ line integration events of *Hermes* in *S. calcitrans* reported in these experiments occurred through a canonical cut-and-paste mechanism. To date, noncanonical transpositions of *Hermes* have been described only in nematocera.

Hermes integration events were recovered from two of the approximately fifty *S. calcitrans* germ lines tested. This frequency of integration is comparable to the *Hermes* integration frequency measured in the germ line of *A. aegypti* (Jasinskiene *et al*., 1998), *C. capitata* (K. Michel *et al*. in preparation), *T. castenaeum* (Berghammer *et al*., 1999a) and *Culex quinquifasciatus* (M. L. Allen *et al.* in preparation). In both of the germ lines from which transgenics were recovered, multiple copies of integrated *Hermes* elements were found. From the germ line of G_0 male #23 we subsequently detected two events and from the germ line of G_0 male #40, a total of five events were detected. Two of the integrated elements from male #40 were present in both of the lines subsequently derived from it. In addition, line 40m3 contained two other events found only in this line, and line 40f18 contained one other unique event. Multiple integrations of *Hermes*, as described here, have been seen in *D. melanogaster, Ae. aegypti* and *C. capitata* (O'Brochta & Atkinson, 1996; Pinkerton *et al*., 2000; K. Michel *et al.* in preparation and unpublished data). The reasons for what appears to be a preponderance of multiple integrations of *Hermes* elements are not clear at this time.

While the present study was not designed to specifically address the question of *Hermes* stability in *S. calcitrans* some observations are relevant to that issue. In the lines derived from G_0 #40, between G_3 and G_8 there appears to be a loss of a band of hybridization. However, the lower molecular weight bands observed at G_8 are disproportionately intense, suggesting the presence of a doublet. Consequently, there may not have been the net loss of an element but the change in the position of the element within the genome. Based on these observations it is not possible to say at this time whether the changes in banding patterns observed between G_3 and G_8 are due to element instability, segregation of unlinked elements or recombination. The lines used in this analysis were not started from homozygous individuals and so the question of the stability of *Hermes* in *S. calcitrans* remains an open one. We have looked for the presence of *hAT*-like elements using degenerate PCR-based methods and have failed to detect any sequences resembling *Hermes* transposase. Neither of these observations allows us to rule out the existence of a related element that might destablize *Hermes* in this species. Further experiments would be needed to test these ideas more thoroughly.

These studies have also revealed an important characteristic of the dominant genetic marker system being used to recognize transgenic nondrosophilid insects. In this and other studies employing *Hermes* as a gene vector, the dominant visible marker EGFP was used. In most cases expression of EGFP was under the regulatory control of the cytoplasmic actin gene (Actin5C) from *D. melanogaster*. When this promoter was tested using transient expression assays in developing embryos of the blow fly *Lucilia cuprina* we found expression levels to rival those of *hsp70* regulated gene expression under identical conditions (Atkinson & O'Brochta, 1992). In addition, transgenic *Ae. aegypti* expressing Actin5C regulated EGFP and EYFP have been created and in both cases expression in the larval and pupal stages was widely distributed and at a high level as indicated by the intensely green or yellow larvae (Pinkerton *et al*., 2000 and unpublished data). Expression of Actin5C-regulated EGFP in *S. calcitrans*, on the other hand, was quite different. In the transgenic lines described here the pattern of expression was distinctly nonuniform and at a low level. Furthermore, the penetrance of the EGFP phenotype was less than 100% despite the presence of multiple copies of the transgene. The characteristic of the expression patterns of the marker gene appears not to be due solely to position effects since line 40m3 had at least four elements and still had a very restricted pattern of EGFP expression. Incomplete penetrance and expressivity were also reflected in the fact that 40m3 and 40f18 had different patterns of expression despite having originated from the same G_0 adult and having multiple *Hermes* elements in common. In light of these results it would appear that alternative promoters such as the one from the polyubiquitin gene of *D. melanogaster* or perhaps one isolated from *S. calcitrans* might be more effective (Lee *et al*., 1988; Handler *et al*., 1998; Handler & Harrell, 1999). Given the less than optimal performance of the genetic marker used to recognize transgenic individuals in this study it is quite possible that transgenic G_1 animals were overlooked and consequently our estimates of integration rates represent underestimates.

Hermes continues to show mobility characteristics that support its continued use as a nondrosophilid insect gene vector. In the case of *S. calcitrans*, as it is for *Ae. aegypti* and other mosquitoes, the ability to generate transgenic

insects is limited as much by difficulties in producing fertile adult G_0s following microinjection as by the specific mobility characteristics of the vector. Improved DNA delivery methods and more efficient animal husbandry methods are sorely needed. Nonetheless, although still a technical challenge, *S. calcitrans* transformation is possible and this will facilitate numerous analyses of this species including the physiology of the midgut and the specific role of gut-secreted antimicrobial peptides (Lehane & Lehane, 1997).

Experimental procedures

Stomoxys calcitrans

The insects used in this study are a wild-type strain collected originally from Hertfordshire, UK and maintained in the laboratory for more than 25 years. Flies were cultured in the laboratory at 24–26 °C, 60% RH in a 12 h light : 12 h dark cycle. Larvae were reared in a mixture of 0.5 litre each of bran, hardwood chips and 'Fibre Provider' (Spillers™ – a mixture of hay, straw and molasses used as horse feed) to which is added one tablespoon of malt extract, 3 g of brewers yeast tablets and 1 litre of water. This mixture is allowed to ferment for at least 3 days before use. Adults in the colony were fed daily on cotton wool swabs soaked with heparinized pig blood. Under these conditions the lifecycle took about 18 days. Females produce eggs about 5 days after eclosion and live for up to 30 days.

Testing Hermes *mobility*

The mobility of *Hermes* in *S. calcitrans* was tested using a modification of the method described by Sarkar *et al.* (1997a). *Stomoxys calcitrans* eggs were collected by placing a cage containing gravid adult females on its side on top of a black cloth such that eggs deposited by females would fall through the mesh of the cage and on to the cloth. Females have a tendency to retain eggs which means that some eggs (estimated to be 15%) prepared for injection are too old to permit transformation. To minimize (but not eliminate) this problem, three to five drops of 10% ammonia were placed on the egg-laying cloth beneath the fly cage and the flies, previously starved for 24 h, were fed blood. The eggs produced in the next 30 min were discarded. Eggs were then collected at 30 min intervals and allowed to mature for a further 30 min at 20 °C. A cage of 500 adult flies will continue to produce sufficient eggs throughout the day for injection of 1000 or more embryos. Eggs aged 30–60 min from laying were dechorionated by soaking briefly (2–3 min) in 50% bleach and washed extensively with water containing 0.02% Triton X-100 before rinsing in distilled water. Very thorough washing in Triton X-100 is necessary or the eggs fail to hatch. The eggs were blotted to remove excess liquid and aligned on double-sided cellotape. Eggs were desiccated briefly (time varied but was always less than 10 min) by placing the mounted eggs in a desiccation chamber containing fresh desiccant. Desiccated eggs were covered with halocarbon oil (Series 700, Sigma, St. Louis, Missouri) and injected with a mixture of DNA containing the appropriate mobility-indicator plasmids.

The mixture of DNA used to assess the mobility of *Hermes* consisted of three plasmids. The first contained a nonautonomous *Hermes* element carrying a kanamycin resistance gene, the *Bacillus subtilis sucraseB* gene, the alpha peptide-coding region of the *E. coli B-galactosidase* gene and the ColE1 origin of replication (pBSHermesKanSacori-lacZ; 0.25 mg/ml). The second plasmid, pHSHH1.9 (0.25 mg/ml), contained the *Hermes* transposase open reading frame under the regulatory control of the *D. melanogaster hsp70* promoter. The third plasmid, pGDV1 (0.5 mg/ml), a chloramphenicol resistance-encoding plasmid from *B. subtilis* incapable of replicating in *E. coli*, served as a target for integration. Injected embryos were allowed to develop from 1 to 2 days at 25 °C in a humid, oxygen-saturated atmosphere after which time viable embryos (as indicated by advanced embryonic development) were collected and plasmid DNA isolated as described (O'Brochta & Handler, 1988). The isolated plasmids were screened for the presence of recombinants arising from transpositional recombination by transforming *E. coli* and selecting for the unique combination of markers expected to arise from such an event, e.g. a chloramphenicol and kanamycin resistant plasmid capable of replicating in *E. coli* and expressing the alpha peptide of *B. galactosidase* and *sucrase*. Plasmid DNA from *E. coli* surviving the selection for transpositional recombinants was analysed further by restriction mapping using the restriction endonuclease *Sal*I. Further confirmation was obtained by DNA sequence analysis with the aid of an ABI 373 automated sequencer (PE Applied Biosystems).

Producing transgenics

The plasmid pHermes(A5CEGFP) (0.15 mg/ml) containing a nonautonomous *Hermes* element with the Enhanced Green Fluorescent Protein (EGFP) gene (Clontech Laboratories Inc., Palo Alto, California) under the regulatory control of the promoter from the *D. melanogaster* cytoplasmic actin gene (Actin5C) (Pinkerton *et al.*, 2000) and pHSHH1.9 (0.125 mg/ml) (O'Brochta *et al.*, 1996) were injected into the posterior pole of preblastoderm embryos as described above. Injected embryos were treated as described above until hatching. Hatched G_0 larvae were transferred in batches to larval diet at a density of 200 larvae per 250 ml of medium. G_0 pupae were collected when dark brown and transferred to tubes 2 inches high and 1 inch wide with gauze at each end and held in a humid chamber. After eclosion the sex of the fly was determined and a virgin wild-type fly of the opposite sex added to the tube. Flies were fed blood once a day by placing a cotton wool swab soaked with heparinized pig blood on top of the tube and eggs were laid through the gauze at the bottom of the tube. Eggs were transferred to water, and 48 h later hatched G_1 larvae were screened for the expression of EGFP using a Leica MZFL III fluorescence stereomicroscope equipped with a GFP2 filter.

Transformed (EGFP) G_1 larvae were transferred to larval diet. The resulting G_1 pupae were collected and isolated as above, allowed to eclose and the resulting adults mated separately to noninjected, virgin wild-type insects as described above. Eggs from the G_2 generation were collected, hatched and larvae screened as described. Separate lines were established with the EGFP larvae arising from each G_2 fly producing transformed larvae. Transformed G_2 larvae were transferred to larval diet and for each subsequent generation (G_3-G_8) of each line all pupae were placed in the same cage and subsequent adults allowed to interbreed. Because of the low penetrance of the EGFP phenotype at each generation we selected only insects with visible expression of EGFP at the first instar to continue the line. Normally 100 EGFP first instar larvae were collected from each line.

Analysis of transgenics

To obtain sufficient genomic DNA from each of the established lines unselected larvae were reared to adults. These adults were

snap frozen and ground to a fine powder in liquid nitrogen and genomic DNA was extracted using the Holmes-Bonner method (Holmes & Bonner, 1973). Ten micrograms of genomic DNA were digested to completion with either *Eco*RI or *Pst*I and size fractionated in a 0.7% agarose gel prepared with Tris-acetate, EDTA buffer (Sambrook *et al.*, 1989). The DNA was transferred to a nylon filter (Hybond-N, Amersham Pharmacia Inc., Piscataway, New Jersey) and immobilized by crosslinking with ultraviolet light (UV Stratalinker, Stratagene, La Jolla, California). Filters were prehybidized at 60 °C for 8 h in 50 mM HEPES pH 7, 3 × SSC, 1% SDS, 10 × Denhardt's solution, 1 mM EDTA, 0.2 mg/ml denatured salmon sperm DNA. Filters were hybridized in fresh prehybridization mix containing the probe under the same conditions for 16 h. Probes were prepared using a random priming method (DECAprime II) in the presence of alpha ^{32}P-dCTP according to the manufacturer's instructions (Ambion Inc., Austin, Texas). Hybridized filters were washed in 2 × SSC, 0.1% SDS at 60 °C for 3–5 h. Patterns of hybridization were detected using a Storm 860 Phosphoimager (Molecular Dynamics Inc., Sunnyvale, California) and analysed using ImageQuant software (Molecular Dynamics).

Inverse PCR analysis was performed by digesting 1 μg of genomic DNA to completion with *Sau* 3A. Digested DNA was treated with T4 DNA ligase in a reaction volume of 200 μl at 16 °C overnight. The resulting circularized DNA was recovered using the GENECLEAN process (Bio 101, Vista, California) and resuspended in 20 μl dH_2O and 1 μl was used in a 40-μl PCR reaction containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM each of dATP, dTTP, dGTP and dCTP; 2 units *Taq* Polymerase (PE Applied Biosystems); 0.495 μM of each primer. A GeneAmp PCR System 9600 thermocycler (PE Applied Biosystems, Norwalk, Conneticut) was used for all reactions. Primers for the analysis of the left end of *Hermes* were 235F (5′-CAG TCG CCT GCC TTA TGC TTT TGG AGA GCG -3′) and 66R (5′-AAT GAA TTT TTT GTT CAA GTG GCA AAG CAC-3′). The right end of *Hermes* was analysed using primers 2681F (5′-AAA ATA CTT GCA CTC AAA AGG CTT GAC ACC-3′) and 2621R (5′-GAG TAT TTT TTC ACA ACT TAA CAA CAA CAG-3′). Reactions were performed under the following conditions; 95 °C (3 min), thirty cycles of 95 °C (15 s), 65 °C (15 s), 72 °C (1 min), followed by 5 min at 72 °C. Reaction products were analysed on a 1% agarose gel containing 5 μg/ml ethidium bromide. The PCR products were cloned using the Original TA Cloning Kit (Invitrogen Corporation/NOVEX®, Carlsbad, California), according to the manufacture's specifications and sequenced with the aid of an ABI 373 automated sequencer (PE Applied Biosystems).

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