

An Analysis of the *hobo* Transposable Element for Gene-Vector Development

Alfred M. Handler and Sheilachu P. Gomez

Insect Attractants, Behavior and Basic Biology, Research Laboratory, USDA-ARS,
Gainesville, Florida

INTRODUCTION

The absence of gene vectors for germline transformation of non-drosophilid insects remains a major barrier to both applied and basic advances in the molecular genetic analysis of insects (Handler and O'Brochta 1991). In order to develop gene vectors that will allow the efficient transformation of insects of agricultural and medical importance, we have focused on transposon-based vectors due to their current use in *Drosophila melanogaster*, as well as their considerable potential as tools for genetic analysis and manipulation, which will be invaluable to the genetic analysis of non-drosophilids. These methods include transposon-tagging (Searles et al. 1982), enhancer-trapping (Bellen et al. 1989), and site-directed gene conversion or gene replacement (Gloor et al. 1992).

Presently, routine and efficient gene transformation of insects is limited to *D. melanogaster*, relying primarily on *P* element-based gene vectors (Rubin and Spradling 1982). These vectors have thus far failed to mediate gene transfer in non-drosophilid insects, and our previous research indicates that *P*-element based vectors will not be useful in non-drosophilids in the near future (Handler et al. 1993). In an effort to develop other transposon-based vectors, we have considered the *hobo* transposon system (see Blackman and Gelbart 1989 for a review) which, like *P*, was initially discovered and developed into a transformation vector in *D. melanogaster* (Blackman et al. 1989). *Hobo* shares general regions of amino acid sequence homology with the plant transposons, *Activator* from maize, and *Tam3* from the snapdragon, suggesting that *hobo* is part of a broadly distributed family of transposable elements (Calvi et al. 1991).

Evidence presented here indicates that *hobo*-like elements also exist in a broad range of insects, and unlike *P*, *hobo* retains mobility properties in all species in which it has been tested, including tephritid fruit flies. This suggests that either *hobo* itself may function as a gene vector in a variety of insects, or that the *hobo*-like elements may be developed into vectors. The data also indicate that *hobo* mobility occurs with or without *hobo* transposase throughout the genus *Drosophila*, but fidelity of the excision process is enhanced by functional *hobo*. This has implications for the possibility of horizontal transmission of *hobo* and thus, its phylogenetic distribution.

METHODS AND MATERIALS

Insect strains. *Drosophila melanogaster* strains known to contain *hobo* (H strains) included the wild type Oregon-R and *Bc Elp/CyO*, P[ry⁺, HBL1]; *ry* (referred to as *hobbled* or *hbl*; Calvi et al. 1991). This is a transformant strain containing a *P*-mediated integration of a *hobo* element having the 3' terminus deleted. A *D. melanogaster* strain known not to contain *hobo* (E strain) is *cn*; *ry*⁴². Other *Drosophila* species were obtained from the Bowling Green collection. Of these only *D. simulans* is known to contain *hobo*. *Anastrepha suspensa* (Loew) and *Toxotrypana curvicauda* Gerstaecker were collected in the wild in Florida. *Ceratitis capitata* (Wiedemann), *Bactrocera dorsalis* (Hendel), and *Bactrocera cucurbitae* (Coquillett) strains were kindly provided by Drs. Stephen Saul and Susan McCombs, University of Hawaii, who also provided use of their laboratory to perform excision assays. Frozen pupae were provided for other molecular analyses.

Excision assays. Similar to our previous analysis of *P* function in non-drosophilid insects (O'Brochta and Handler 1988, O'Brochta et al. 1991, Handler et al. 1993) we developed an *in vivo* transient expression total excision assay to assess *hobo* mobility, rapidly and quantitatively, in insect embryos (Figure 1). The *hobo* excision assay consists of an indicator plasmid having a reporter gene (*lacZ*) within a non-functional *hobo* element, and a helper plasmid encoding *hobo* transposase, being injected into insect embryos. Functionality of the transposase gene in the host embryo is determined by *hobo* excision from the indicator plasmid, which deletes the *lacZ* reporter gene.

Indicator plasmids were constructed by having the entire *hobo* element within the *KpnI*-*SstI* fragment in pHFL1 (Calvi et al. 1991) inserted into the pK19 (kanamycin resistant) cloning site (Pridmore 1986). The *lacZ* α peptide gene from pUC19 was inserted into the pHFL1 *NdeI*-*ScaI* site creating pKHFL*lacZ*. The indicator plasmid was co-injected into embryos with the pSH2 (ampicillin resistant) helper plasmid which has the *hobo* open reading frame under *hsp70* promoter regulation. In some experiments the unmodified *hobo* gene within pHFL1 was used as a helper plasmid (pHFL1 and pSH2 kindly provided by B. Calvi and W. Gelbart, Harvard University). Pre-blastoderm embryos were injected with helper: indicator plasmid concentrations of 0.3:1.0 mg/ml injection buffer (5 mM KCl, 0.1 mM PO₄, pH 6.8) or 1.0 mg/ml indicator plasmid alone under halocarbon oil with an air-pulse injection system. Subsequently the embryos were incubated in an oxygenated environmental chamber. After 16-20 hr incubation and heat shock, plasmids were harvested from surviving embryos and transformed into bacteria by electroporation. Bacteria were plated on X-gal, kanamycin media allowing only indicator plasmid-transformed bacteria to survive. A lack of excision maintains the *lacZ* reporter gene resulting in β -galactosidase activity in transformed bacteria, which results in blue colonies on X-gal media. Both precise and imprecise *hobo* excisions from the indicator plasmid result in the loss of *lacZ*, yielding white bacterial colonies. White or light blue colonies were re-streaked and plasmid DNA prepared for further analysis. Putative excision plasmids were analyzed by restriction digests and in some cases sequence analysis.

Final excision frequencies were computed by dividing the total number of white colonies (*LacZ*⁻) yielding excised indicator plasmid by the total number of colonies from a minimum of three separate injection experiments. Total indicator plasmids assayed for each strain tested ranged between 50,000 and 100,000.

Nucleic acid analyses. *hobo*-related sequences were amplified from genomic DNA using polymerase chain reaction (PCR) using indicated primers and typical reaction conditions (Sambrook et al. 1989) with cycling parameters of initial denaturation at 94°C for 1 min, followed by 35 cycles of 1 min at 93°C for denaturation, 1 min at 50°C for annealing, and 2 min at 72°C for extension, with a final extension for 10 min. Amplified products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Amplified sequences were subcloned into T-nucleotide overhang vectors and sequenced using Sequenase (US Biochemicals) based protocols.

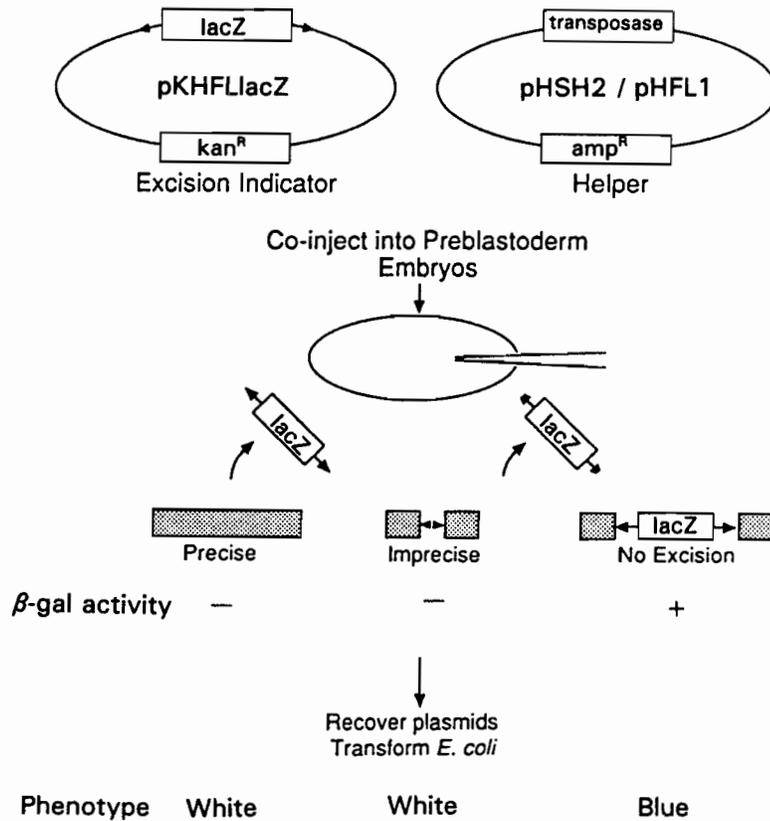


Figure 1. The *in vivo hobo* excision assay using the pKHFLlacZ indicator plasmid including the lacZ α peptide reporter gene inserted into the *hobo* open-reading frame. *hobo* transposase is provided by a heat shock promoted or unmodified *hobo* gene within a separate helper plasmid, or by chromosomal copies of *hobo* within the host insect genome. See Methods for further details.

Southern analysis was achieved by digesting *A. suspensa* and *B. dorsalis* genomic DNA with *Xho*I or *Hind*III and separating the fragments by 0.7% agarose gel electrophoresis. DNA was transferred to nylon membranes, UV-irradiated and hybridized to ³²P-labelled probe (subcloned PCR *hobo*-like sequence).

RESULTS

hobo Mobility in Drosophilids

Initial assays were performed in the *Drosophila melanogaster* *cn; ry* E strain, devoid of *hobo* elements, with the pKHFLlacZ indicator plasmid and either the heat shock regulated *hobo* transposase (pHSH2) helper or the unmodified *hobo* gene helper (pHFL1). Figure 2 shows that *hobo* excision events were not detected without coinjection of a helper plasmid, but with pHSH2 helper, an excision frequency of approximately 0.8×10^{-3} /pKHFLlacZ indicator plasmid resulted. Use of the

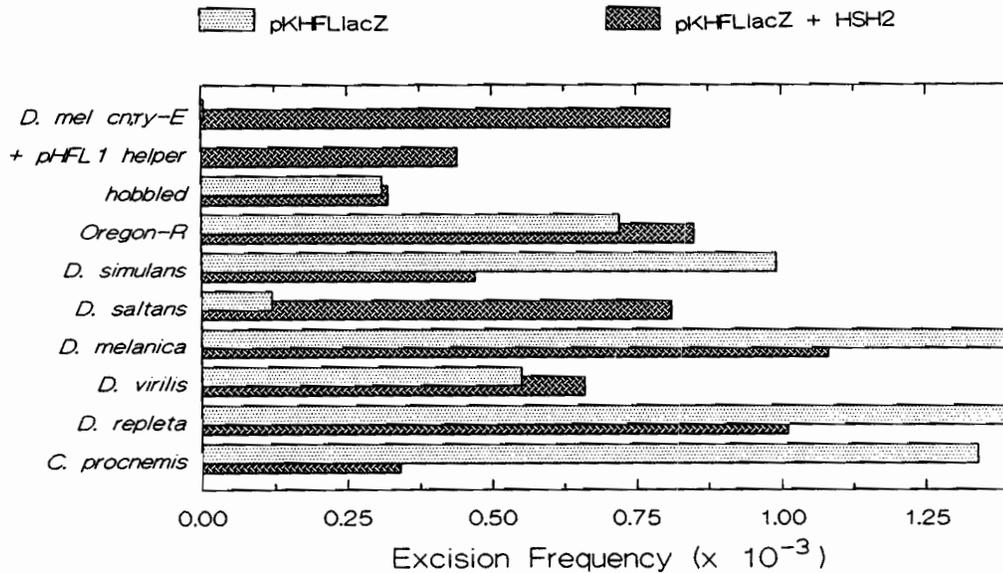
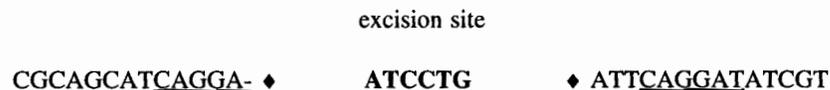


Figure 2. *hobo* excision frequencies in *D. melanogaster* strains and *Drosophila* species with and without helper plasmid. pHFL helper indicates that pHFL1 was coinjected with pKHFLlacZ indicator plasmids into the *D. melanogaster cn;ry*⁴² strain. In all other strains the HSH2 helper was coinjected with indicator plasmid and resultant excision frequencies are designated by the cross-hatched bars. Frequencies with indicator plasmid injected alone are designated by the stippled bars. Frequencies are the compiled data from three or more independent injection experiments with 4-10 x 10⁴ total indicator plasmids assayed per experiment.

unmodified pHFL1 helper also resulted in excision, although at a 50% lower level, indicating that *hobo* is functional in somatic tissue as well as in the germline. In the strains known to have functional chromosomal *hobo* elements, including Oregon-R, *hobbled*, and *D. simulans*, excision occurred at significant rates with or without helper plasmid.

Interestingly, in drosophilid species known not to contain *hobo* (Daniels et al. 1990), excision also occurred in the absence, as well as in the presence of *hobo* helper. Higher rates of excision without helper occurred in *D. melanica*, *D. repleta*, *Chymomyza procnemis*, as well as *D. simulans*, suggesting a negative interaction between injected *hobo* and the resident system(s). Only in *D. saltans* was excision substantially lower without helper.

Sequence analysis of excision products in *D. melanogaster cn;ry* and *D. virilis* revealed the major excision event facilitated by functional *hobo* (use of helper) in both species to be a nearly precise excision (occurring at the *hobo* termini; ♦) with addition of an 8 bp inverted duplication (in bold) of proximal chromosomal insertion site DNA (underlined).



Several other similar nearly precise excision events occurred, but with varying amounts of duplicated or additional DNA incorporated at the excision site. In both species a small number of imprecise excisions with breakpoints within *hobo* or surrounding chromosomal DNA also occurred in the presence of *hobo* transposase (not shown). In contrast, in *D. virilis* all the sequenced excisions

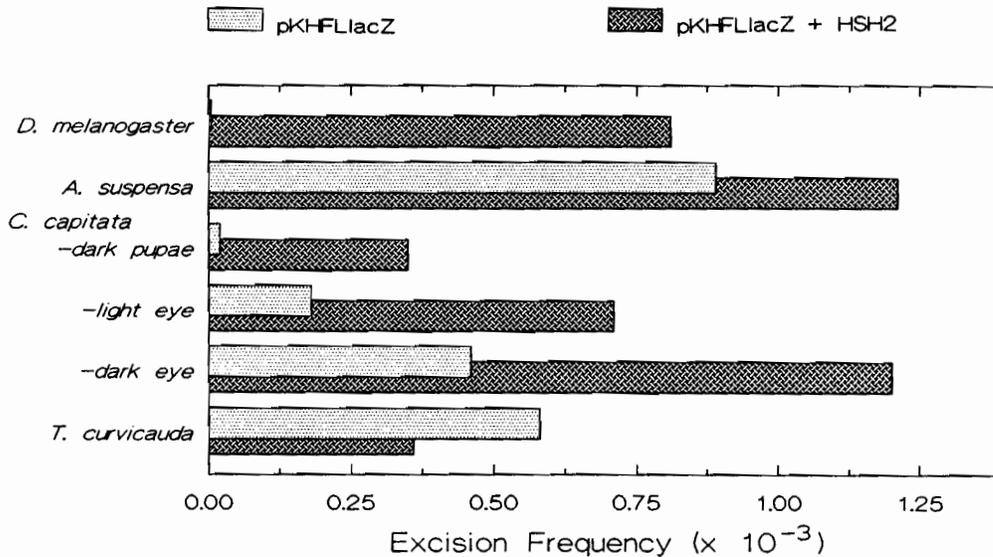


Figure 3. *hobo* excision frequencies in tephritid species and with and without helper plasmid. Frequencies resulting from coinjection of the pKHFLlacZ indicator and pSHH2 helper plasmids are designated by the cross-hatched bars. Frequencies with indicator plasmid injected alone are designated by the stippled bars. Frequencies are the compiled data from three or more independent injection experiments with $4-10 \times 10^4$ total indicator plasmids assayed per experiment

that occurred without *hobo* helper were imprecise ($n=12$), having breakpoints within *hobo* and/or in the chromosomal or pK19 vector DNA. Several plasmids did not sequence presumably due to loss of the pK19 priming site.

The data indicate that cross-mobilizable systems, perhaps related to *hobo*, exist in a broad range of drosophilids. In distantly related *D. virilis*, excision in the presence of *hobo* transposase is functionally similar to *D. melanogaster*, and in both, the excision process resembles that of the related plant transposons *Activator* (Federoff 1989) and *Tam3* (Sommer et al. 1985) in terms of the duplication of insertion site DNA at the excision site. In the absence of *hobo* the excision process is consistently random or undirected, suggesting that the putative cross-mobilizable system in *D. virilis* is not functionally identical to *hobo*.

***hobo* Mobility in Tephritid Fruit Flies**

Hobo excision assays were performed in several tephritid species using the pKHFLlacZ indicator plasmid and the pSHH2 helper (Figure 3). In the Caribbean fruit fly, *Anastrepha suspensa*, and the papaya fruit fly, *Toxotrypana curvicauda*, excision frequencies were similar in the presence or absence of helper, although excision was more frequent in *A. suspensa*. Three mutant strains of the Mediterranean fruit fly, *Ceratitidis capitata*, were also tested. In two strains, *light eye* and *dark eye*, excision also occurred with and without helper, although in both strains excision frequencies were three-fold higher with exogenous helper. In the *dark pupae* strain however, a very low level of excision occurred without helper, with a six-fold higher level of excision occurring with helper.

The excision assays indicate that all three tephritid species are able to support *hobo* mobility, albeit at somewhat varying levels. This is in contrast to similar studies with the *P*-element, whose

mobility was restricted to drosophilid species (O'Brochta and Handler 1988, Handler et al. 1993). Similar to the various species tested, most of the tephritid strains were autonomous in their ability to support mobility without exogenous helper. This suggests that tephritid fruit flies may also harbor *hobo*-related elements able to cross-mobilize *hobo*. Unfortunately, the presence of a cross-mobilizable system would make these strains poor candidates as hosts for germline transformation since it is likely that integrations would be unstable (negative regulation of transposition might also occur). It is therefore encouraging that the *C. capitata dark pupae* strain apparently requires exogenous helper similar to the *D. melanogaster cn; ry E* strain, suggesting that it may also be devoid of *hobo* or a cross-mobilizable system, and therefore a useful host strain to test *hobo*-mediated transformation.

***hobo*-Like Elements**

To identify *hobo* or *hobo*-like elements (HLE) in insects, DNA was amplified from the genomic DNA of various insects. PCR primers were made to DNA sequences within the *hobo* transcriptional unit which encode four to five amino acid sequences identical to those found in *Activator* as determined by the similarity analyses of Calvi et al. (1991). Two highly conserved primers, 991For and 1428Rev (primer names designate the primer orientation and 5' nucleotide primer position in the pHFL1 *hobo* element; see Calvi et al. 1991) amplified the intervening DNA sequence in both *hobo* and *Activator*, and amplified genomic DNA sequences from various *Drosophila* species, *Anastrepha suspensa* and *A. striata*, *Helicoverpa zea*, *Heliothis virescens*, *Musca domestica*, and *Stomoxys calcitrans*. Primers pairs 991For-1428Rev, as well as 991For-2229Rev and 1407For-2229Rev (yielding overlapping and contiguous sequences, respectively) were also used for gene amplification of genomic DNA from 31 mutant and wild type strains of the tephritids *C. capitata*, *B. dorsalis* (Oriental fruit fly), and *B. cucurbitae* (melon fly). The data summarized in Table 1 show that sequences were amplified in many, but not all of the strains of both *C. capitata* and *B. dorsalis*, while no fragments were detected in the three *B. cucurbitae* strains. This indicates that HLEs are well distributed, but may not be universal in these species. Further functional and structural testing of the negative strains will determine whether they are truly devoid of HLEs.

Given our primary interest in tephritid flies, the single 991For-1428Rev fragments (approx. 450 bp) generated in wild type *A. suspensa* and wild and mutant strains of *C. capitata* and *B. dorsalis*, were subcloned, sequenced, and compared to the corresponding 438 bp sequence in *hobo*. After sequence alignment, an approximate 50-60% homology was revealed between *hobo* and the tephritid sequences. Genomic southern blots using the respective *hobo*-like PCR fragments as probe indicated that the elements exist in multiple copies at various sites in the genome which is supportive of them being part of mobile elements. Efforts are in progress to isolate the complete elements from genomic libraries.

DISCUSSION

The functional and physical data presented indicate that several drosophilid and tephritid species are permissive for *hobo* mobility and that the tephritid species harbor *hobo*-related elements. Similar to the findings of Atkinson et al. (1993) in *Musca*, *hobo* excision occurred in the absence, as well as in the presence of *hobo* transposase in both insect families. This suggests that cross-mobilizable systems, perhaps related to *hobo*, exist in a broad range of insects.

In *D. melanogaster* and distantly related *D. virilis*, excision in the presence of *hobo* transposase is functionally similar occurring primarily at the *hobo* termini, while in the absence of *hobo* in *D. virilis*, the excision process is consistently random or undirected. This suggests that the putative cross-mobilizable system in *D. virilis* is not functionally identical to *hobo*, or that other species-

Table 1. PCR analysis of tephritid strains using *hobo* sequence primers.

Tephritid Strain	PCR Fragment		
	991For -1428Rev	991For -2229Rev	1407For -2229Rev
<i>Ceratitis capitata</i>			
white eye	++	+	++
brown orange	++	++	+
apricot	(+)	++	(+)
orange	+	++	-
MED	(+)	++	-
red	-	+	-
light eye/double chaete	-	+	-
wild type-Rossler	-	-	-
M. kula	-	-	-
C mass	-	-	-
brown orange/dark pupae	-	-	-
dark eye	-	-	-
dark pupae	-	-	-
<i>Bactrocera dorsalis</i>			
Alt x DOR	++	+	-
white eye	++	(+)	-
DORTS	++	-	-
matte	++	-	-
Kahuku	++	-	-
amethyst	++	-	-
Grape	++	-	-
Sakamoto	++	-	-
Furrow	++	-	-
curled	(+)	-	-
small wing	++	-	-
Ahe Ahe	+	-	-
B. dorsalis Kauai	-	-	-
melanistic	-	-	-
Mandarin red	-	-	-
<i>Bactrocera cucurbitae</i>			
Cue	-	-	-
wing bubble	-	-	-
light eye-white	-	-	-

- = no visible PCR signal

(+) = faint signal

+ = clearly visible signal

++ = strong to very strong signal

specific cellular factors may influence excision. This result supports the notion that both autonomous and non-autonomous *hobo* elements can be transmitted horizontally into other species; however, it may be argued that the non-autonomous class is more subject to deletion and rearrangement in species distantly related to *D. melanogaster*. This possibility, in addition to possible negative interactions between *hobo* and the resident cross-mobilizable system (or other cellular factors), may explain, in part, the narrow phylogenetic distribution of *hobo* (Daniels et al. 1990, Pascual and Periquet 1991, Simmons 1992). Isolation of the *hobo*-related systems allowing direct tests of their activity and interaction with *hobo* should clarify these possibilities.

The apparent lack of restriction on *hobo* mobility in distantly related drosophilids and tephritids suggests that *hobo* may be useful as a transformation vector in these species. Though fidelity of the transposition process promoted by *hobo* transposase may be maintained in *D. virilis*, and thus probably most drosophilids, it remains undetermined whether fidelity is maintained in tephritids and other distantly related insects. Results from Atkinson et al. (1993) with *Musca domestica* and preliminary evidence with *A. suspensa* from our lab (unpublished) suggest that this is not the case, since in these species *hobo* transposase did not appear to catalyze nearly precise excision events. A lack of fidelity in the excision process would make *hobo* an inefficient or unreliable vector, requiring the development of the *hobo*-like elements (or other transposons) in the species of interest, into gene vectors. Nevertheless, the general presence of these elements (a lack of "E" strains) resulting in cross-mobilization activity, may preclude stable integration of *hobo* or *hobo*-related vectors. Thus, a priority for future research should be the identification of tephritid strains devoid of *hobo* or *hobo*-like activities. In this respect, the *C. capitata* dark pupae strain is of considerable interest as a host strain for germline transformation since, thus far, it remains refractory to amplification of *hobo*-like elements and it supports only an extremely low mobilization of *hobo* in excision assay tests. Additional putative E strains may be similarly identified by testing *hobo* mobility in the remaining strains which do not yield *hobo*-related amplified DNA.

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