

A Functional Analysis of the *P*-Element Gene-Transfer Vector in Insects

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A *P*-element mobility excision assay was used to determine if non-drosophilid insects could support *P* gene vector function. Present studies included the testing of Muscids, Sphaerocerids, and Phorids, none of which were able to support *P* mobility. A new excision indicator plasmid was developed allowing the detection and recovery of virtually all *P*-element excision products. The frequency and sequence analysis of excision products from *Drosophila melanogaster* and another drosophilid, *Chymomyza procnemis*, indicated both quantitative and qualitative differences in the activity of transposase. The quantitative relationships observed in the original assay were maintained, and qualitative differences in transposase activity were reflected in the sequence of the empty donor sites. The results suggest that host factors are involved in cutting and ligating *P*-element DNA during excision, with transposase facilitating these processes. Possible limitations on *P* mobility by abnormal transposase transcript processing were tested in *Anastrepha suspensa* using transposase-encoding plasmids having deleted intron sequences. A transposase cDNA supported normal *P* excision in *D. melanogaster*, and a low level of mobility in *A. suspensa*. Possible applications of gene transfer in insects are presented, in particular methods to genetically sterilize and sex insects for the sterile-insect technique. © 1993 Wiley-Liss, Inc.

Key words: gene transformation, non-drosophilid, *Drosophila melanogaster*, *Anastrepha suspensa*

INTRODUCTION

The use of molecular genetics in its full potential to understand the biology of insects and control their population size will depend on the ability to transfer recombinant DNA molecules modified in vitro, into the genome of a

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host insect. Gene transfer is presently possible, as a routine and efficient method, only in *Drosophila melanogaster* using gene vectors derived from *D. melanogaster* transposable elements. The use of these gene vectors, particularly *P*-element based vectors, in non-drosophilid insects has not resulted in vector-mediated integration events [1–3]. Unfortunately, the gene transformation experiments themselves have given few hints as to the limitations involved, and clearly, the complexity of the method makes a systematic analysis by gene transformation experiments prohibitively difficult [4]. Of all the major factors involved in gene transfer, including the introduction of DNA into germ cells, the ability of DNA to integrate into a host chromosome, and the efficient selection of transformants, we have considered DNA integration to be the major limiting factor.

In terms of *P*-element based vectors, the failure to integrate is likely due to a restriction in the normal mobility properties of the transposon in a heterologous system. In order to assess the mobility properties of *P*, and possibly other gene vectors in insect embryos, we have developed transient expression assays based upon *P* excision from plasmids injected into preblastoderm embryos [5]. These assays test the ability of the *P*-encoded transposase to be produced and function, as well as, more generally, the ability of the embryonic milieu to support *P* movement. In addition to defining specific limitations on *P* mobility, the assays also allow modifications, which might overcome such limitations, to be tested in a systematic and quantifiable manner.

We have found that the frequency of *P* excision is a function of an insect's relatedness to *D. melanogaster*, and is almost totally restricted outside of the Drosophilidae. A major limitation is apparently a failure in proper RNA transcript processing, although a transposase cDNA helper is limited in its ability to catalyze *P* excision. The necessity for non-*P*-encoded factors present in *Drosophila* for *P* mobility is presently being considered.

MATERIALS AND METHODS

In Vivo Excision Assay

The *P* excision assays were performed essentially as described previously [5]. Briefly, the frequency of *P* mobility was assessed by monitoring gene function restoration following *P*-element excision from a plasmid-encoded gene (Fig. 1). Excision assay indicator plasmids (pISP and pISP-2) which were constructed by Rio et al. [6] have a small non-autonomous *P*-element sequence surrounded by white gene DNA inserted into the lacZ α peptide coding region of pUC8. In this configuration, lacZ α , which is required in appropriate bacterial hosts for β -galactosidase activity, is nonfunctional. In the presence of functional transposase and any other required host factors, the *P* element can be mobilized to excise resulting in a restoration of lacZ α function. Excision assay plasmids and plasmids encoding somatically active transposase (pUCHs π Δ 2-3) were injected into preblastoderm insect embryos (within 2 h of oviposition) and incubated 18–24 h. The plasmids were subsequently harvested as low molecular weight DNA and transformed into appropriate bacteria. Bacterial colonies transformed with plasmids having restored lacZ α

P Excision Assay

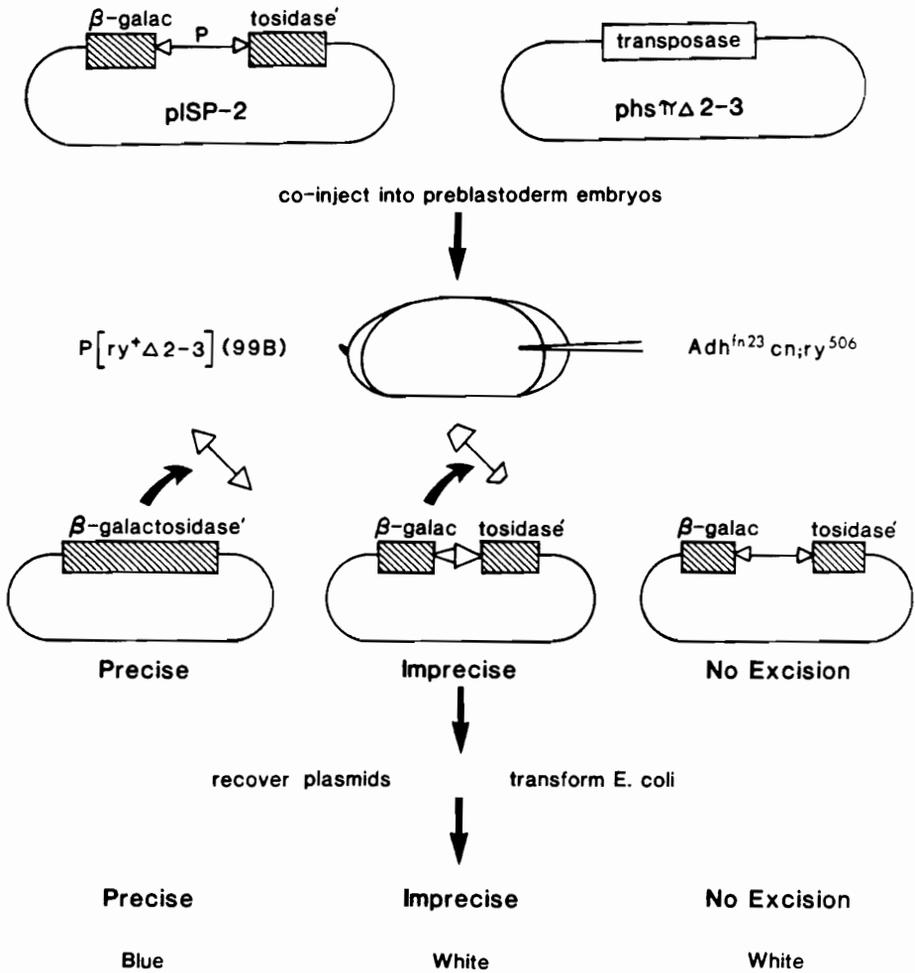


Fig. 1. P-excision assay using the pISP-2 indicator plasmid. In $P[ry^+\Delta 2-3](99B)$ embryos, having a chromosomal source of somatically active transposase, only the indicator plasmid is injected. Methods are described in the text.

activity due to precise or nearly precise P excision express β -galactosidase activity and have blue coloration when grown on X-gal media. A lack of excision, or "imprecise" excision, fails to restore *lacZ α* function resulting in white bacterial colonies. The frequency of excision is the relationship between the number of blue to white colonies.

Total Excision Assay

In order to monitor all excision events (imprecise as well as precise excision) we modified the original indicator plasmids (pISP-2) by inserting the *Es-*

cherichia coli S12 ribosomal protein gene into the *P* sequence [7]. S12 acts dominantly to confer sensitivity to streptomycin in streptomycin-resistant bacteria [8] and thus the new indicator plasmid ($p\pi\text{strep}^S$) permits all *P* excisions to be monitored by virtue of a streptomycin-resistance phenotype. The excision process can be further defined by the ability to restore *lacZ* α function. A nonbiased sampling of excision products was then analyzed by DNA sequencing using Sequenase™ (U.S. Biochemicals, Cleveland, OH) according to the manufacturer's specifications.

Sources of Transposase

Excision activity was usually catalyzed by a somatically active transposase gene encoded on the plasmid $p\text{UCHs}\pi\Delta 2-3$ [9], which contains the transposase gene under *hsp70* promoter control and having the intron 3 sequence deleted. In some experiments transposase was provided chromosomally from a stable integration of an intron 3 deleted *P* element found in the strain $P[ry^+ \Delta 2-3](99B)$ [10]. Other *P*-element helpers tested were $p\text{UCHs}\pi\Delta 1-2-3$ and $p\text{UCHs}\pi\text{cDNA}$, having introns 2 and 3 and all introns (1, 2, and 3) deleted, respectively (all of the above plasmids provided by D. Rio, Whitehead Inst., Cambridge, MA). The $p\text{UCHs}\pi\text{cDNA}$ was found to have a base deletion which was reverted by oligonucleotide site-directed mutagenesis (according to Sambrook et al. [11]) to create a new plasmid, $p\text{Khs}\pi\text{cDNA}$. RNA was generated in vitro from some of the helper constructs by placing them under SP6 promoter control. Synthetic capped transcripts were produced using SP6 RNA polymerase (Promega, Madison, WI) according to manufacturer's specifications. *D. melanogaster* embryonic nuclear extract was purchased from Promega (Madison, WI).

Other Plasmids

The influence of specific *P* sequences, or the products they encode, on *P* mobility was tested. Test plasmids included $p\text{hs}\pi$ [12] which has the entire transposase transcriptional unit under *hsp70* control, and $p\text{Carnegie4}$ [13], which is a *P* gene vector containing only the terminal sequences of *P*.

RESULTS

Monitoring "Precise" Excisions of *P*

To further define the phylogenetic limits of *P* function we extended our initial in vivo analysis of *P* mobility in various non-drosophilid insects, using an embryonic *P*-element excision assay [5]. We found that all drosophilids tested supported *P* mobility, though the frequency of excision decreased as a function of relatedness to *D. melanogaster* (Fig. 2). Most of the non-drosophilid species tested, including Muscids, Phorids, Sphaerocerids, and two tephritid species, failed to support *P* excision. Preliminary experiments with one non-drosophilid, *Paralimna decipiens* (family: Ephydriidae), indicated that this species could support a low level of *P* excision; however, these results have not been confirmed due to difficulty in rearing Ephydrids. These results confirm our earlier conclusion that *P* mobility, and probably the current *P*

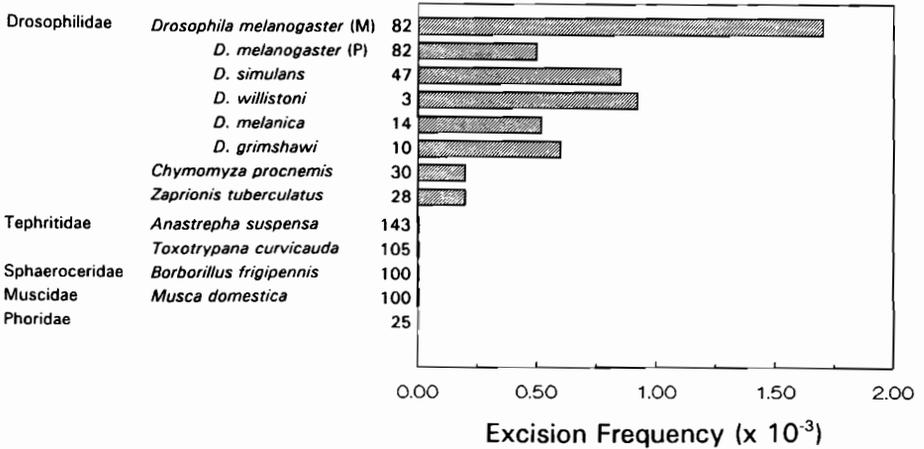


Fig. 2. Excision frequencies in drosophilid and non-drosophilid insect species determined by the *P*-excision assay. Numbers beside the bars indicate thousands of pISP plasmids screened.

gene vector system, is phylogenetically restricted to drosophilids and closely related families.

Monitoring All Excisions of *P*

The original excision assay only revealed those excision events ("precise") which allow expression of the lacZ α reporter gene. Yet genetic analyses in *D. melanogaster* have indicated that precise excisions of *P* resulting in mutant reversion are probably a minority of all excisions [14]. Thus the original excision assay provides only a limited assessment of *P* mobility. Indeed, the lack of detectable excision in non-drosophilids may represent a qualitative and not a quantitative difference in transposase function due to imprecise excisions which do not result in phenotype reversion. In order to test this possibility and to more critically assess *P* mobility, we developed a new indicator plasmid, π strep^s, containing the bacterial streptomycin-sensitivity gene within the *P* sequence, permitting all excision events to be monitored.

We have used this new *P* excision assay to compare the ability of distantly related drosophilids, *D. melanogaster* and *Chymomyza procnemis*, to support *P* mobility [7]. Comparable quantitative differences in the excision frequencies relative to the original assay were seen between the species (Table 1). Both the total and precise excision frequencies in *Chymomyza* were approximately 10% of those observed in *Drosophila*. However, in both species approximately 40% of all the excisions resulted in a reversion of the *P*-induced mutant phenotype. Transposase-independent excision was also observed in both species. The new assay supports our previous conclusion that *P* excision is phylogenetically restricted, resulting in a reduction of both precise and imprecise excision. The recovery of transposase-independent excision products suggests the involvement of host-encoded factors in excision with *P* transposase mediating or facilitating the process.

TABLE 1. Excision Products Assayed With the Total Excision Assay Using the P π strep^s Indicator Plasmid

	<i>D. melanogaster</i>		<i>C. procnemis</i>	
	Freq. ($\times 10^{-3}$)	(n) ^a	Freq. ($\times 10^{-3}$)	(n)
Total excisions	5.5	(74)	0.47	(327)
Precise excisions	1.9	(74)	0.20	(327)
Transposase independent excisions	0.06	(230)	0.06	(71)

^an = Number of thousands of pISP plasmids screened.

Analysis of excision reaction products. The total excision assay allows almost a total recovery of *P*-excision reaction products, allowing comparative analyses. Sequence analysis revealed qualitative differences in transposase activity in *D. melanogaster* and *C. procnemis*, further indicating that the host environment (or factors) can influence the way transposase mediates *P* excision (Fig. 3). In both species most excision breakpoints were internal to *P* leaving up to 8 nucleotides behind; however, the actual excision breakpoints showed species specificity. In *C. procnemis* the number of the excisions having one breakpoint in sequences flanking *P* was more than twice the number found in *D. melanogaster*. In addition, the analysis suggests that 3' and 5' breakpoints are not chosen independently. Qualitative differences in the excision reaction products may reflect differences in the activity of host-en-

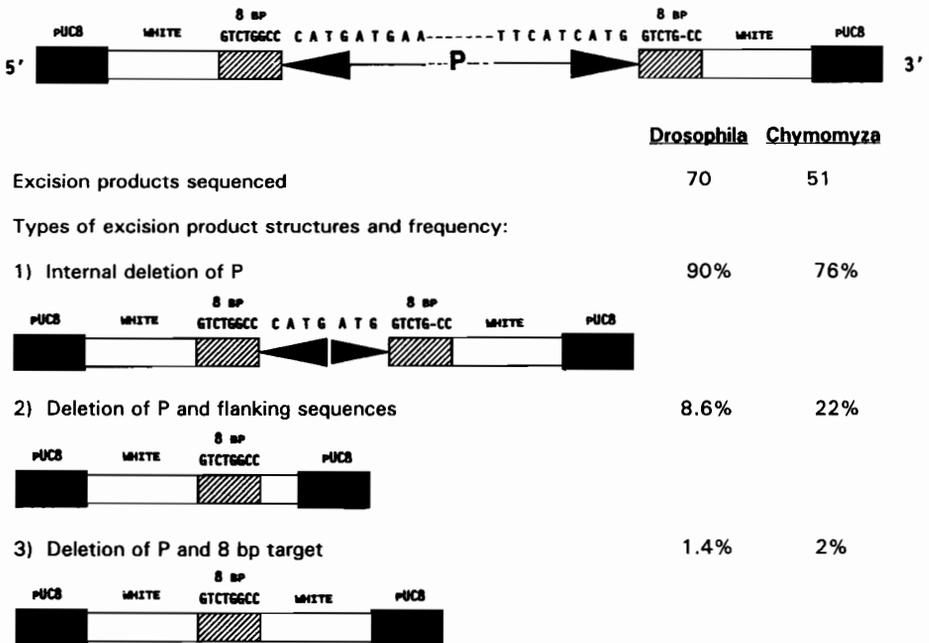


Fig. 3. Frequencies of classes of excision products determined by sequencing of P π strep^s indicator plasmids after *P*-element excision in *Drosophila melanogaster* or *Chymomyza procnemis*. The schematic represents *P*-element terminal sequences and flanking sequences proximal to both termini.

coded co-factor(s), whose involvement in *P* mobility has previously been inferred from DNA binding experiments [15].

Transposase Transcript Processing

Intron deleted transposase helpers. In *D. melanogaster*, splicing of intron 3 occurs only in the germline and is apparently the basis of the tissue specificity of excision and transposition [9]. The generation of transcripts from constructs in which intron 3 has been deleted results in the production of functional transposase, capable of driving excision in *D. melanogaster* somatic tissue. In an effort to determine whether *P* mobility is restricted in other insects due to incomplete or abnormal transcript processing we tested the ability of modified forms of the transposase gene, and in some cases transposase RNA generated in vitro from these genes, to promote *P* excision. In addition to the transposase gene $hs\pi 2-3$ (lacking intron 3), which was used in our initial tests of *P* mobility, we tested $hs\pi\Delta 1-2-3$ (lacking introns 2 and 3) and $hsp\pi cDNA$ (lacking all introns), as well as transposase transcripts generated in vitro from pSP6 $hs\pi\Delta 2-3$ plasmids (Table 2). In *D. melanogaster* all of the transposase helper molecules supported *P* excision, though the influence of SP6 $hs\pi\Delta 2-3$ RNA was considerably less compared to the other helpers. Notably, while helpers having introns 2 and 3 deleted (pUCH $hs\pi\Delta 2-3$, pUCH $hs\pi\Delta 1-2-3$, and pSP6 $hs\pi\Delta 2-3$ RNA) failed to support *P* excision in the tephritid *A. suspensa*, excision was supported by a cDNA helper having all introns deleted. Initial tests with the $hsp\pi cDNA$ in *A. suspensa* indicated an excision frequency of 7×10^{-5} . While this frequency is approximately twentyfold lower relative to *D. melanogaster*, this is the first indication of *P* excision in a species distantly related to *Drosophila*.

The low frequency of excision in *A. suspensa* with the cDNA helper may be due to the lack of requisite cofactors in non-drosophilids. To test this possibility crude nuclear extracts from *D. melanogaster* embryos were co-injected with transposase cDNA in excision assays. Interestingly, the nuclear extract had an inhibitory influence on *P* excision in *Drosophila*, decreasing excision by approximately one-third (Table 2). In *Anastrepha*, however, the nuclear extract increased excision by about 50% above excision using only the cDNA. If inhibition in *Drosophila* represents a general negative effect of the extract, then the positive influence of specific factors in *Anastrepha* might actually be substantially greater.

TABLE 2. Influence of Various Helper Molecules on *P* Excision

Helper molecule	Excision frequency ($\times 10^{-3}$) ^a	
	<i>D. melanogaster</i>	<i>A. suspensa</i>
pUCH $hs\pi\Delta 2-3$ DNA	1.70	0
SP6 $\pi\Delta 2-3$ RNA	0.25	nd ^b
pUC $\pi\Delta 1-2-3$ DNA	2.10	0
pKh $hsp\pi cDNA$ DNA	1.40	0.07
pKh $hsp\pi cDNA$ + nuclear extracts	0.97	0.12

^a30–100,000 pISP plasmids screened per helper tested.

^bnd = not determined.

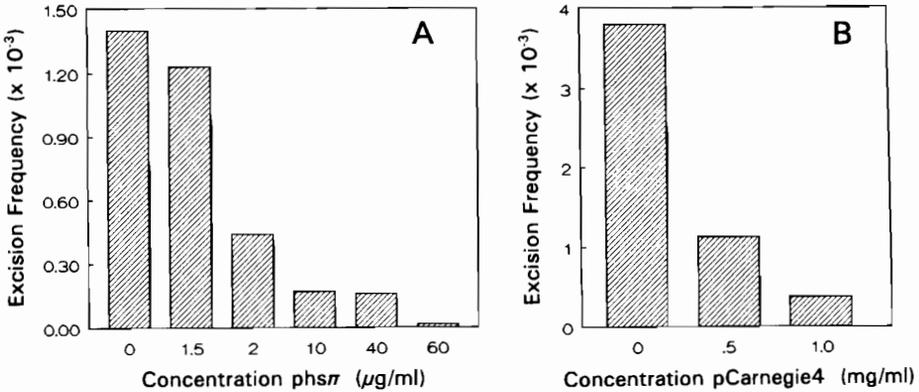


Fig. 4. Excision frequencies in *Adh^{fn23} cn; ry⁵⁰⁶* embryos injected with 0.5 mg/ml pUChs π Δ 2-3 and pISP-2, and indicated concentrations of (A) phs π or (B) pCarnegie4. For each sample 50–100,000 pISP plasmids were screened.

Detection and Analysis of *P*-Element Repressors

Alternative *P*-encoded transcription products, as well as *P*-element sequences, have been suggested as repressors of *P* mobility (see Engels [16]). The *P*-excision assays provide a unique way to detect and analyze *P* repressor activity by their coinjection with appropriate indicator and helper plasmids. We found that the somatic expression of a transposase gene (phs π) containing all three introns can completely eliminate *P* excision in embryonic somatic cells at very low concentrations relative to pUChs π Δ 2-3 (Fig. 4A). Because processing of the third intron does not occur in the soma, phs π is expected to result in incompletely processed transcripts giving rise to truncated polypeptides lacking transposase activity. Presumably the truncated transposase product can bind to *P*, but cannot catalyze its mobility. Repression may be due to competition for binding between the functional and nonfunctional transposase products. These results suggest that inaccurate transcription or translation of the transposase gene in non-drosophilid systems could result in the production of repressors.

P mobility was also repressed by the presence of pCarnegie4 plasmids which contain *P*-element terminal sequences containing the presumptive transposase binding sites (Fig. 4B). However, repression occurred only at concentrations similar to that of the active binding site present in pISP-2. Presumably the binding sites present on the nonfunctional *P* element act to bind, and therefore deplete the amount of transposase available for catalyzing mobility of the functional *P* element.

DISCUSSION

We had previously found that *P* mobility was restricted outside of the Drosophilidae family, and that among drosophilids *P* mobility was a function of relatedness to *D. melanogaster* [5]. Based upon these findings we concluded that the use of present *P*-element based gene vectors to catalyze gene trans-

formation in non-drosophilid insects would be highly inefficient, if not impossible. To further define the phylogenetic restrictions on *P* mobility, we have extended these studies by testing other dipteran species. Evidence is also presented indicating the basis of restrictions on *P* mobility and possible means of ameliorating them.

Tests with the original excision assay indicated a lack of *P* mobility in several additional insect families including Muscids, Sphaerocerids, and Phorids. The possibility remained, however, that the results with the original assay actually indicated qualitative differences in the excision process as opposed to differences in the ability to support excision due to the detection of only precise excision events which resulted in the restoration of a functional lacZ α peptide. Nevertheless, the quantitative results with the original assay were supported, and expanded upon, with the new total excision indicator plasmid which allows almost all excision events to be assayed. In comparing *P* mobility in *Drosophila melanogaster* and *Chymomyza procnemis*, the total excision frequency in *D. melanogaster* was approximately tenfold higher than in *C. procnemis*, although in both species only about 35–40% of all excision events resulted in a reversion to the *P*-induced mutant phenotype (Table 1). Thus the quantitative relationship between the species for both total and precise excisions remained the same for both assays. A low equivalent level of transposase-independent excision was also observed in both species suggesting that non-transposase factors are involved, if not required. Sequence analysis of the reaction products recovered from the two species revealed qualitative differences in transposase activity. Although sharing similar breakpoints internal to *P*, there were differences in the relative frequencies of particular breakpoints. In addition, *C. procnemis* had a greater than twofold higher percentage of excisions with breakpoints in sequences flanking *P*. Taken together these results suggest that: 1) transposase may not be directly involved in the cleavage process, but rather may facilitate it; 2) the cleavage events occur interdependently, and 3) in addition to the frequency, the fidelity of the excision process is also lessened in species other than *D. melanogaster*, possibly due to differences in non-*P*-encoded factors. As will be discussed below, the discovery and use of such factors may be critical to the function of *P* vectors in non-drosophilids.

P mobility is regulated in *D. melanogaster*, at least in part, by differential intron-splicing, and it was realized early on that aberrant transcript processing might be involved in restricting transposase function in other species. Although this has not been directly determined in insects, transposase RNA is known not to be spliced in plants [17]. A lack of or inefficient processing in insects is consistent with the preliminary experiments reported here showing that a transposase cDNA helper functions in a tephritid fruit fly. Although *P* mobility was twentyfold lower compared to *D. melanogaster*, if this relationship carries over to gene transformation frequencies, the cDNA helper might be useful for those insects which can be easily injected. We are encouraged to begin testing the cDNA helper; however, reliable and rapid selection systems for non-drosophilid transformants remains a major limitation.

We inferred previously that non-*P*-encoded factors may be involved in the fidelity of the excision process; however, the same or other factors may also

influence the frequency of excision and/or transposition. This possibility is supported by the preliminary observation that the transposase cDNA does not attain full function in a non-drosophilid insect, but that function is increased by the addition of factors present in *Drosophila* nuclear extracts. A present goal is to discover and isolate such factors. The excision assays provide a useful system to achieve this by allowing the systematic testing of proteins and RNA from *Drosophila* embryos by their coinjection into non-drosophilid embryos.

Uses of Gene Transformation

Basic and applied uses of gene transformation in non-drosophilid insects have been discussed previously [4], though some significant applications should be cited here. Based on studies in *D. melanogaster*, the most straightforward uses of gene transformation will come from the basic information gained about insects as it relates to gene regulation and the genetic influences on reproduction, development, behavior, and evolution. These studies will significantly broaden our understanding of insect biology, and in turn enhance current methods, and help in developing new methods to control insect populations. Some of this information might be gained by currently available molecular biological techniques such as somatic extrachromosomal expression, transfection into cell lines, and transformation of insect genes into *D. melanogaster*. Nevertheless, these techniques all have inherent limitations, and critical analyses of gene activity and structure-function relationships will require the transfer of manipulated DNA back into the genome from where it came. In terms of applied and field applications for biological control, stable gene transformation will be essential.

Our immediate focus on potential field applications of gene transformation will be on male sterilization and selection for the sterile-insect technique [18]. Sterilization might be achieved by placing dominant-acting neomorphic genes, possibly isolated from *D. melanogaster*, under conditional control. Once in an insect genome, under nonpermissive conditions such genes could act to disrupt some aspect of male reproduction. Under permissive conditions, the sterility gene would be nonfunctional (or act normally) allowing a breeding stock to be maintained. Information gained from genetic analyses in *D. melanogaster* also presents a number of novel ways of using manipulated DNA to genetically select for a particular sex. The most simplistic method is to place a gene that encodes a selectable product under sex-specific regulatory promoter control. Unfortunately, we know in *D. melanogaster* that many sex-specific promoter regions affect other controls (e.g., tissue and temporal) [19] which would make the system inefficient. A more promising approach, but more speculative for non-drosophilids, is the use of sex-specific intron splicing mechanisms currently observed in *D. melanogaster* sex-determination genes [20]. This would allow the maintenance of an in-frame transcriptional unit in only one sex. For example, an intron with sex-specific alternative 3' splice sites could be linked to a toxin coding region (i.e., diphtheria toxin subunit A). Expression of the lethal toxin would only occur in females, thereby selecting for males. A much more speculative approach would be manipulation of sex-determination genes themselves. In *D. melanogaster* a temperature-sensi-

tive allele of the *transformer-2* sex-determination mutant (*tra-2^{ts}*) exists which, at the nonpermissive temperature of 29°C, results in both the XX chromosomal females and the XY males developing into sterile males [21]. At permissive temperature, genetically identical siblings develop normally into reproductively competent insects. It is likely that *tra-2^{ts}* represents the most ideal genetic-sexing and sterilization strain possible, making full use of all zygotes with minimal manipulation beyond temperature regulation. Although the isolation and manipulation of this particular mutant strain has not required molecular techniques in *D. melanogaster*, molecular methods will certainly be required to find, test, and utilize analogous genes in other insects.

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