

# PROSPECTS FOR GENE TRANSFORMATION IN INSECTS<sup>1</sup>

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KEY WORDS: germline transformation, gene vectors, P-element mobility, transposons

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## PERSPECTIVES AND OVERVIEW

The ability to manipulate genetic material *in vitro* and integrate it into a host genome has proven to be one of the more powerful methods of genetic analysis, as well as a means to manipulate an organism's biology. In insects, the use of gene transformation is equally significant in its potential to facilitate an understanding of insect genetics, biochemistry, development, and behavior. A more complete understanding of insect biology would in turn certainly enhance current methods, and promote development of new methods, to manage populations of both beneficial and pest species. Despite the benefits to be derived from gene-transfer, the routine and efficient introduction of exogenous DNA into insect genomes is limited to the genus *Drosophila*. Although DNA has been integrated into the genomes of three mosquito species (76, 80, 86), this integration has apparently resulted from rare random integration events, and the utility of this method is uncertain. The inability to achieve routine gene transfer in insects therefore makes the nature

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of this review more prospective than retrospective. Current methods allow the *in vivo* analysis of manipulated insect genes, such as by extrachromosomal transient expression (somatic transformation) (70, 72) or integration of genes from nondrosophilid species into *Drosophila* (83). While these methods are useful for specific applications in some insects, they are inherently limited in terms of the types of genes that can be analyzed and the scope of analyses possible (42).

The primary method of gene transformation in *Drosophila* has utilized gene vectors derived from the P transposable element that has inherent mobility properties. Initially, the transposition of P observed in drosophilids distantly related to *D. melanogaster* (8), which do not normally contain P, suggested that P might be phylogenetically unrestricted and therefore useful as a gene vector in other insects. The development of P vectors allowing the dominant selection of neomycin-resistant transformed insects (111) permitted the testing of this notion. However, testing of P gene vectors in several nondrosophilid species, using both germline and somatic transformation tests, has thus far revealed a lack of P-vector function in these insects.

Given that P-element-based gene vectors in their present state are either nonfunctional or very inefficient in nondrosophilid insects, attempts to modify the P-element system or to develop new gene vectors or methodologies for gene transfer must be considered. Modification of the P-element requires methods that will allow a further understanding of normal P function in *Drosophila*, and a testing of P modifications in heterologous systems. The development of new gene vectors will require the isolation of new transposable elements and an evaluation of their ability to serve as vectors. In some organisms, DNA with mobility properties has been revealed using hybrid dysgenic-induced mutations and restriction fragment length polymorphisms, but these methods are most efficient in species that have been well characterized genetically and are easily subject to molecular studies. In addition, some, if not most, of the transposons identified with these methods will not fit the criteria for gene vector development, or, as with P, their use may be restricted to a few related species. Clearly, most current approaches to gene vector development are not trivial and may prove especially difficult for use in particular species.

Another general approach to gene-transfer may utilize random integration of DNA into chromosomes by nonhomologous recombination. This is routinely used in mammalian systems (37), and some success has been reported in various insect species. However, the relatively low frequency of random integration in insects would suggest that for this technique to be efficient, methods for mass introduction of DNA into germ cells and mass selection for integration will be required.

Beyond development of a means to integrate exogenous DNA, it has become clear that improvements in DNA delivery systems and selection

schemes for integration are required. Indeed, while the P-element system has reportedly failed in nondrosophilids, low survival rates and inconsistencies in the selection schemes used actually make a critical evaluation impossible. In addition, a lack of recovery of transformants could result from limitations in the amounts or stability of the DNA delivered. A proper evaluation of these points is essential before a critical consideration of vector systems can be made.

## APPLICATIONS OF GENE TRANSFORMATION

### *Basic Applications*

The use of gene transformation in a wide variety of organisms has already demonstrated the vast amount of biological information that might be gained with such techniques (37, 47, 117). Of particular importance to insects are the various aspects of genetic analysis that have been impeded because of difficulties in cytogenetics, individual mating, rearing, and long generation times, among others. Transformation techniques coupled with *in vitro* DNA manipulation should prove invaluable to the identification and isolation of insect genes and to defining their structure-function relationships. While some of this information may be derived from transfection in insect cell lines or transient extrachromosomal expression, clearly the interactive influences of specific genetic components during developmental processes require whole animal germline transformation.

**TRANSPOSON TAGGING** Genes are generally identified in most organisms by mutational analysis. In the absence of localized mutant alleles, genes can be isolated from genomic or cDNA libraries as a function of their homology to previously isolated genes or their transcriptional activity. Many insects are not amenable to mutational analysis, and the range of genes that can be isolated as described is somewhat limited. Transposon-based gene vectors can overcome these limitations because many show little site specificity when they integrate into a genome, and as a result integration can often disrupt a normal gene function, leading to a mutant phenotype (see 64). If the disrupted gene is of interest based upon its mutant phenotype, it can be subsequently localized and isolated using the transposon DNA with which it is tagged for chromosome *in situ* hybridization or to probe an appropriate library. Transposon tagging has been used to isolate dozens of genes from *D. melanogaster* (56) and similar applications using mammalian retroviral gene vectors have been used to identify and isolate genes involved in mammalian development (108).

**MUTANT RESCUE** An efficient gene transformation system could also be used to isolate genes without relying on tagging methods, as well as to confirm the identity of putative clones. This may be achieved by integrating

cloned DNA containing a putative wild-type gene into the genome of a mutant animal. Rescue or complementation of the mutant phenotype would confirm the presence of the functional wild-type gene within the vector. More precise localization of the gene within the cloned DNA can then be accomplished by systematically deleting DNA followed by successive rounds of gene transformation. This method has been used effectively to identify and isolate genes from *D. melanogaster*, but only after their position had been established within the order of tens or hundreds of kilobases because the number of germline transformants that can be generated is limited. For *Drosophila*, this limitation is directly related to the number of embryos that can be injected. If efficient methods for introducing vector DNA into large numbers of insects existed in conjunction with an efficient system for selecting for vector integration, then entire genomic libraries could be introduced by gene transformation methods (95). This method would permit gene isolation by mutant rescue to be implemented with virtually any gene without prior localization as is currently necessary.

**ENHANCER TRAPS** The identification and isolation of genes using mutagenesis and tagging methods has been very fruitful. Yet these methods may exclude genes showing pleiotropy or a lethal phenotype. These limitations have been overcome in *Drosophila* by a method called *enhancer trapping*, which relies on the ability of relatively strong regulatory sequences within the genome to promote the activity of vector-encoded sequences inserted nearby. This method was originally developed to identify bacterial transcription units and to determine their orientation (13). Recently, a similar scheme was developed in *D. melanogaster* using the *Escherichia coli*  $\beta$ -galactosidase gene under the promoter control of the weak P-element promoter (2, 92, 118). The activity of this promoter is low enough so that  $\beta$ -galactosidase activity does not reach levels detectable by whole-tissue histochemical staining. If, however, the element transposes into a site adjacent to a transcriptional enhancer, the activity of the fusion gene will be increased and  $\beta$ -galactosidase activity will be detected. The pattern of expression revealed histochemically allows one to determine if the enhancer identified is associated with a gene involved in a developmental process of interest. This strategy has revealed a number of developmentally regulated loci and will undoubtedly find routine use in the future.

**GENE REPLACEMENT** Most gene transfer methods in eukaryotes result in a gene insertion into a nonhomologous site within the genome without affecting the resident homologous allele. This complicates some analyses when the expression of the resident allele interferes with the function or assessment of the introduced gene. Such a problem is minimized when the resident gene is a

recessive null mutant allele whose activity may be superseded by an introduced functional allele. Most insects, however, have few mutant strains or null alleles. This limitation may be overcome by the actual replacement of the resident gene with an *in vitro* modified copy. In addition, gene insertion into an unusual genomic site may result in a position effect that alters normal gene expression. For enhancer traps, this effect is desirable; however, often the alteration confuses the analysis of a particular gene or gene-construct. Gene replacement would ameliorate such position effects by allowing integration into the normal genetic milieu.

Gene replacement is routinely accomplished in yeast (47) and has been demonstrated in plants (94), mammalian cell lines (114), and slime molds (21). These events depend on homologous recombination between DNA within the vector and the chromosome, and are quite rare. The successes mentioned have resulted largely from powerful selection schemes that can be imposed on large numbers of cells *in vitro*.

### *Field Applications*

Since the one insect shown to be amenable to gene transformation, *D. melanogaster*, is of limited agricultural or medical importance, the potential field applications of gene-transfer remain somewhat more prospective. Nevertheless, at least the basic biological information to be gained as it relates to chemical resistance mechanisms, sex determination, hybrid sterility, and hormone action and metabolism will enhance current insect management programs and will help develop new ones. One particular biological control method that might rapidly take advantage of gene-transfer techniques is the sterile-male release program (62), which could be greatly enhanced with efficient means of genetically sexing and sterilizing males. Classical genetic techniques, generally using a selectable gene linked to a male-specific Y chromosome, have undergone considerable study. Although some success has been achieved using chemical resistance to select males in mosquitoes (105), other genetic-sexing strains have proven highly susceptible to breakdown resulting from chromosome instability, recombination, and mutant reversions. (30, 48). Molecular techniques could minimize these difficulties by creating relatively small chimeric genes that have a selectable gene-product coding region linked to a sex-specific regulatory region. The genetic damage due to transformation would be negligible compared to chromosome rearrangements and mutation induction required by classical techniques. Implementation of such schemes may be easier than for other field applications using transformed insects because most strategies would allow the containment of genetically altered breeding parental insects, limiting release to sterile-male progeny.

## STRATEGIES FOR TRANSFORMING ORGANISMS

The production of transgenic plants and animals has relied on a variety of methods. The simplest methods are perhaps the least understood from a mechanistic standpoint and involve the direct introduction of DNA, usually as a plasmid, into a plant or animal cell nucleus. The DNA, once present in the nucleus, randomly integrates into the host's genome by an illegitimate recombination event. This method is used to transform insect and noninsect cell lines and to create transgenic mice, plants, nematodes, and slime molds. The frequency of transformation using this method can be quite high. For example 20–30% of the mouse oocytes injected with DNA will result in a transgenic mouse (37). However, a disadvantage of this method is that often the DNA integrates as multiple copies oriented in a head to tail fashion (15), which can complicate subsequent analysis of the transformants. The random integration of DNA into insect genomes has been observed, although more data are necessary to properly evaluate frequencies and possible mechanisms (34, 76, 80, 86).

Improvements in the integration frequency can be achieved by physically linking the DNA to be integrated to vector DNA with inherent mobility properties. Vectors currently used to transform the germline of organisms have usually been derived from either retroviruses such as amphotropic murine leukemia viruses (37), transposons such as the P-element from *Drosophila* (27), or infectious agents such as the Ti plasmid from *Agrobacterium tumefaciens* (117). A variety of nonretroviral vectors have been developed for mammalian and insect cell lines but these are not useful for whole animal transformation.

### *Gene Transformation in Drosophila*

Early attempts were made to genetically transform *D. melanogaster* by soaking mutant embryos in solutions of wild-type genomic DNA (31, 32). A variety of somatic mosaics resulted, but these had no clear inheritance of completely reverted phenotypes. The conclusion was that genetic transformation had occurred as a result of episomal transmission and not chromosomal integration. Subsequent attempts to revert *vermillion* mutant lines by injection of wild-type DNA met with some success (34, 69), although this result was not repeated and integration was never verified biochemically. The efficient and routine integration of DNA into a *Drosophila* host genome awaited the development of gene vectors created from modified P-element transposons (102, 103), and more recently, the *hobo* element (6). As in other organisms, these gene vectors have taken advantage of the inherent mobility properties of transposable elements. Although we know that transposable elements comprise a significant proportion of the *Drosophila* genome, not all transposons

are conducive to gene vector activity. Early consideration was given to retroviruslike elements such as *copia*, but their relatively low mobility made them poor candidates for gene vectors (96).

An appreciation of how the P gene vectors function in *Drosophila*, possible restrictions on their function in other insects, and how this system may serve as a model for new gene vectors requires an understanding of P biology [see Engels (27) for an extensive review]. Furthermore, since *Drosophila* is the only insect subject to routine gene transformation, a review of the methodology can illuminate some of the special problems and factors to be considered in transforming insects.

**P-ELEMENT VECTORS** The P-element is a highly mobile transposon discovered by virtue of the genetic defects that resulted from an induction of its mobility in interstrain crosses of *D. melanogaster*. These collective defects, including elevated mutation rates, chromosome rearrangements, sterility, and male recombination, are known as hybrid dysgenesis (57). The ability of an autonomous 2.9-kilobase (kb) P-element to transpose depends upon the integrity of its 31-base pair (bp) terminal inverted repeats and an internal transcription unit composed of four open reading frames encoding an 87-kilodalton (kd) protein (54, 91). This protein, or *transposase*, is required for both P excision and transposition, although its precise mechanistic role is unknown. At least two levels of control regulate P-element movement, both of which directly affect transposase function. First, P-elements can only be mobilized when present in a poorly defined cellular state known as M cytotype (28, 55). This maternally inherited cellular property is usually found in strains lacking autonomous P-elements. Strains containing autonomous P-elements usually develop a cytotype (P) that represses P movement. The maternally inherited cellular factors that determine cytotype are unknown, though mutated P-element genes may play a role. Second, P-element movement is limited to the germline. Although P-element transcription occurs in somatic and germ cells, complete transcript processing (including splicing of all three introns) required for functional transposase production is germline specific (66). Somatic cells splice only the first two introns, resulting in a truncated nonfunctional transposase product (97).

The development of P-elements into gene vectors was facilitated by their ability to be structurally modified such that foreign DNA can be inserted without destroying their ability to be mobilized (102, 103). If the transposase gene is defective or deleted, mobility can be promoted by transposase provided in trans. Thus in practice, the P gene vector consists of plasmid DNA containing a marker gene surrounded by P terminal sequences but lacking the transposase gene. The vector is mobilized in germline tissue by the presence of an intact transposase (helper) gene located on a separate plasmid, which

promotes P integration into a chromosomal site. Normally, only the P termini and intervening DNA (usually including a selectable marker gene) are integrated. The transposase helper has structurally altered (or deleted) terminal sequences that prevent its integration and subsequent inheritance. Without a source of transposase in succeeding generations, the vector DNA is stably inherited. For unknown reasons, part of the integration process involves creation of an 8-bp duplication of the chromosomal target site (91). Transformation markers have routinely used the wild type genes for *rosy* (103) and *white* eye color (60), *alcohol dehydrogenase* (35), and more recently the bacterial neomycin-resistance gene, neomycin phosphotransferase (111).

The efficiency of P transformation depends on the host used, the size of the vector, concentration of DNA, and the way embryos are handled. Approximately 80% of the embryos can be expected to hatch and 40–50% will reach adulthood following careful injection (95). A variable percentage, ranging from 10–90%, of these adults can be sterile because of genetic background effects or as a result of damage or abnormalities induced by injection. Of the resulting fertile adults, 10–60% can be expected to yield at least one transformed progeny, though very large P vectors such as those containing cosmid inserts can transform with efficiencies 10 times lower (95). This evaluation in *Drosophila* indicates that, using a P vector of moderate size, one should anticipate, as a conservative estimate, recovering at least one transformant following the injection of 100–200 eggs.

Autonomous P-elements have been found only in strains of *D. melanogaster* and *D. willistoni*, though nonmobile P sequences exist in many species of the subgenus *Sophophora* (10, 17, 18, 65). Interestingly, these sequences are not found in some species most closely related to *D. melanogaster* such as *D. simulans* and *D. mauritiana*. Although the distribution of P is discontinuous, P has been mobilized in all drosophilids tested, as shown by gene transformation or embryonic mobility assays (90). P-mediated transformation was reported for *D. simulans* (19, 104) and a distantly related drosophilid, *D. hawaiiensis* (8), at frequencies expected for *D. melanogaster*. However, for *D. hawaiiensis*, only autonomous P elements, and not the altered P vector, were found to integrate, suggesting that in this species P activity is limited.

**HOB0 VECTORS** P-element vectors have been used almost exclusively for *Drosophila* transformation; however, a new transformation vector has been developed recently utilizing the *hobo* transposon. Although *hobo* was originally discovered because of its association with a glue protein gene (75), it was subsequently shown to transpose following certain interstrain crosses, a phenomenon closely paralleling P-M hybrid dysgenesis. While *hobo* shares several structural characteristics with P, notably a 3.0-kb length, 12-bp

terminal inverted repeats, and production of an 8-bp duplication of integration target sequences, it has no sequence similarity with P, and the transposons do not cause cross-mobilization (5).

The *hobo* transformation vector, H[(ry<sup>+</sup>)harl] (6) is analogous to the P vector Carnegie 20 (102) in that it has deleted internal sequences and a *rosy*<sup>+</sup> gene marker. Successful transformation was achieved in both “*hobo* dysgenic” embryos (having resident *hobo* elements), injected with vector alone, and in nondysgenic embryos, injected with vector and a complete *hobo* element helper plasmid (6). The efficiency of transformation in both cases was similar to that of P.

**TRANSFORMATION METHODOLOGY** The generation of transgenic *Drosophila* has become, in the course of just a few years, a routine laboratory procedure. Specific details about the physical operations involved in producing transformants such as preparation of DNA, eggs, needles, and injection procedures have been thoroughly described by others (see 102, 109 for detailed protocols). In brief however, the general procedure involves collecting freshly laid eggs and dechorionating them either manually or chemically in diluted bleach. The eggs are attached to a glass coverslip with double-sided tape and desiccated briefly until they are slightly flaccid. The eggs are then covered with halocarbon oil and those still in the preblastoderm stage are injected at their posterior end with buffer containing the P-element vector and a transposase-producing helper plasmid. Larvae that hatch are placed on regular diet and adults (G<sub>0</sub>) are backcrossed in individual matings. The resulting G<sub>1</sub> progeny are selected or screened for the presence of the marker gene integrated with the P-element, which can be verified by Southern analysis and chromosomal in situ hybridization.

### *Gene Transformation in Nondrosophilids*

Before the initial attempts to transform *Drosophila*, successful somatic gene transformation was first reported in *Ephestia*, in which mutant larvae injected with wild-type DNA gave rise to adults with wild-type wing scales (14). Similar experiments were repeated in *Ephestia* using red eye mutants and in *Bombyx* using *white* mutants treated with wild-type DNA (88). In both experiments, a wild-type adult was recovered, but while the phenotype was inherited, transmission was non-Mendelian. Although one explanation of the unusual inheritance considered “replicating instabilities,” it is more likely that extrachromosomal inheritance occurred.

Interest was regained in the possibility of transforming insects with P transformation in *D. melanogaster*, especially after the demonstration of P transformation in distantly related drosophilids (8) and the development of vectors (i.e. pUCHsneo) containing the dominant-selectable marker gene

neomycin phosphotransferase (NPT) (111). These factors indicated, respectively, that P mobility might not be phylogenetically restricted and that resistance to the neomycin-analog, Geneticin® (G418), could be used in many insect species as a selection for gene integration. The discovery that somatic restrictions on P could be alleviated by deletion of the third intron of the transposase gene (66, 97) also offered the possibility that the resulting P helper, pUChs $\pi\Delta 2-3$ , could produce functional transposase in heterologous systems.

Attempts to transform insects using the pUChs $\pi\Delta 2-3$  helper and pUChsneo vector have been reported for several mosquito species, the Mediterranean fruitfly, *Ceratitis capitata*, and *Locusta migratoria*. In a process similar to the *Drosophila* protocol, these experiments involved injecting plasmids into preblastoderm embryos ( $G_0$  generation), mating the surviving adults, and subjecting their  $G_1$  progeny (or subsequent generations) to G418 selection. For some experiments, putative transformants were subjected to Southern blot analysis to verify and define the nature of the integration event.

**MOSQUITOES** Thus far, the only nondrosophilids in which gene integration has been reported are mosquitoes, but apparently none of these integrations were P mediated. In *Anopheles gambiae*, one chromosomal integration event was detected resulting from 310 surviving  $G_0$  adults mated *inter se* (80). However, Southern blot analysis indicated that this integration resulted from a single recombination event between the host genome and *white* gene sequences on pUChsneo. Similar experiments in *Aedes triseriatus* yielded two patterns of integration from 57 surviving  $G_0$  adults backcrossed to uninjected mosquitoes (76). Curiously, neither integration was consistent with a P-mediated transposition or a simple recombination event as occurred in *A. gambiae*. Furthermore, no subsequent neomycin-resistant survivors showed evidence of chromosomal integration and the precise nature of the integrations originally observed remains obscure. In identical experiments with *Aedes aegypti*, from 71 surviving  $G_0$  adults, one integration event was reported, but as in previous experiments, Southern analysis failed to demonstrate a P-mediated integration (86).

**TEPHRITIDS AND LOCUSTS** Efforts to transform tephritid fruitflies, including the Caribbean fruitfly, *Anastrepha suspensa*, by our laboratory and *C. capitata* by others have failed to recover either P-mediated or random chromosomal integrations. In two experiments on the medfly, a total of over 20,000 embryos have been injected and nearly 900  $G_0$  adults mated (77, 99). In both experiments, G418-resistant  $G_1$  larvae were selected, but resistance was variable or lost in succeeding generations and in no case did Southern blotting show P integration. In one of these experiments, the researchers (77)

inferred that G418-resistance either was inherent in the subpopulations tested or resulted from extrachromosomal expression of the vector-encoded resistance gene. The general lack of persistence of plasmid DNA in *Drosophila* (72) would suggest that the former explanation is more plausible. Similar efforts to transform *L. migratoria* have also failed to reveal a P integration (116).

**LIMITATIONS** The failure to recover germline transformants in heterologous systems by direct application of the *D. melanogaster* P-transformation system could result from a number of limitations including: (a) an inability to transcribe or translate the transposase gene, (b) mobility constraints due to vector size or construction, (c) an inefficient or ineffective selection system, (d) a lack of appropriate genomic target sites, or (e) a lack of requisite host-encoded cofactors or the presence of repressors. Unfortunately, one cannot critically test these various possibilities independent of one another using germline transformation experiments, and yet one must understand the limitations of the system before restrictions can be ameliorated. A common difficulty encountered in the transformation experiments described above was the relatively high frequency of G418-resistant lines recovered that did not have NPT gene integration. This frequency may have resulted from variability in sensitivity to G418 or leakiness in the resistance-phenotype, yet these possibilities are difficult to have controls for. Indeed, the variability in the selection raises the possibility that transformants may have been created but failed to express the resistance phenotype and were lost. Thus, the P-vector system has probably not been fairly evaluated by these experiments. Clearly, effective selection schemes are essential for transformation, and various methods exist other than G418-resistance. Screening for integration is not insurmountable even if it requires direct DNA hybridization. Of more immediate concern is whether the gene vector is capable of transposition.

**EVALUATION OF P MOBILITY** Evaluating the mobility properties of a transposon-based gene vector in heterologous systems depends on first determining the mobility of the transposon independent of other vector DNA, especially selectable markers. Methods should be direct, rapid, quantitative, and preferably allow a systematic testing of modifications. In this respect, we have made efforts to analyze P-element function using excision assays that directly monitor P mobility in the soma of insect embryos. The rationale of this approach is based upon the observation that P-element insertions often result in gene inactivation, and that reversion of these mutations frequently results from the excision of the transposon (39). Since both P-element excision and transposition depend upon normal transposase function, restoration of gene function disrupted by a P insertion can be used to assess P mobility and transposase activity.

Rio et al (97) took advantage of these characteristics of P movement to develop a rapid assay that assesses P function in cell lines. Plasmids (pISP and pISP-2) were constructed that allow the detection of P mobility as a result of gene function restoration following P-element excision from a plasmid-encoded gene. Specifically, a small nonautonomous P-element sequence surrounded by *white* gene DNA was inserted into the *lacZ $\alpha$*  peptide coding region of pUC8. In this configuration, *lacZ $\alpha$* , which is required in appropriate bacterial hosts for  $\beta$ -galactosidase activity, is nonfunctional. In the presence of functional transposase and any other required host factors, the P-element can be mobilized, resulting in a restoration *lacZ $\alpha$*  function. When these plasmids are harvested after incubation in cells and transformed into appropriate bacteria, staining for  $\beta$ -galactosidase activity (blue coloration on X-gal media) acts as an indicator for excision of P from *lacZ $\alpha$* .

We have since modified the in vitro excision assay so that P functionality can be assessed in the insect embryonic soma, enabling us to directly address the question of P mobility in nondrosophilids (see 90 for detailed protocols). In general, the pISP indicator plasmid was injected into preblastoderm embryos in the presence of an endogenous (chromosomal) or exogenous (plasmid) source of transposase. After an overnight incubation, plasmids were recovered and subsequently transformed into bacteria. This assay tests the expression of the transposase gene into a functional gene product, as well as determining more generally whether the embryonic milieu is supportive of P mobility. A limitation of this assay is that the pISP indicator plasmid only permits detection of those excisions restoring *lacZ $\alpha$*  function. Yet these may actually represent only a minority of all excisions because of events that fail to restore the *lacZ $\alpha$*  reading frame. We therefore modified the indicator plasmid by inserting the *E. coli* S12 ribosomal protein gene into the P sequence. S12 acts dominantly to confer sensitivity to streptomycin in streptomycin-resistant bacteria (20) and, thus, the new indicator plasmid (p $\pi$ strep<sup>s</sup>) permits all P excisions to be monitored by virtue of the streptomycin-resistance phenotype. Excision can be further defined by the ability to restore *lacZ $\alpha$*  function.

Thus far, P mobility has been assessed in a variety of drosophilids and nondrosophilids with the original assay (90), and in *D. melanogaster* and *Chymomyza procnemis*, a drosophilid outside the *Drosophila* genus, with the new total excision assay. We found that P could be mobilized in all the drosophilids tested as well as in a closely related ephydrid, *Paralimna decipiens* (D. A. O'Brochta & A. M. Handler, unpublished results). However, the P excision frequency decreased as a function of relatedness to *D. melanogaster*, and P mobility was not detected in tephritids, sphaerocerids, muscids, or phorids. Comparable to the original assay, assays with the new p $\pi$ strep<sup>s</sup> plasmid also showed a 10-fold lower total excision frequency in *C.*

*procnemis* relative to *D. melanogaster*; however, in both species, excisions restoring *lacZ* function accounted for only 30% of the total. The new assay also allows the unbiased recovery of excision products, and sequence analysis indicates qualitative differences between the species in terms of site preference for excision breakpoints.

While these experiments are still in progress, the results thus far indicate that the P-element is not functional in nondrosophilids, and the P gene vector system in its present state would be nonfunctional or highly inefficient in these insects. However, P mobility assays may also be used to define the basis of the dysfunction and to test modifications that may ameliorate it.

**P-ELEMENT DYSFUNCTION** Several explanations for restricted P mobility in insects other than *D. melanogaster* are based on our current knowledge of P-element activity. While the transposase gene is transcribed in *A. suspensa*, Northern analysis indicates a profile of varying-sized transcripts generated from the pUCHs $\pi\Delta 2-3$  helper in embryos (90). This profile may indicate abnormal transcript processing, which could allow termination-codon usage, resulting in a nonfunctional truncated gene-product. At present no direct evidence indicates aberrant processing in insects. A transposase RNA splicing assay, however, using a  $\beta$ -galactosidase reporter gene linked in-frame to the second exon, indicates a lack of, or inefficient splicing of, intron 1 in *Anastrepha* (D. A. O'Brochta, unpublished results). In plants, aberrant processing of P has been shown directly in tobacco transformed with *hs* $\pi\Delta 2-3$ , where abundant 1.5-kb transcript results from transcript termination just beyond the second intron, and intron 1 is not processed (73).

If normal processing is not occurring in nondrosophilid insects, then transposase helpers that have introns 1 and 2 deleted, in addition to intron 3, may be functional. We began testing this notion by using as helpers pUCHs $\pi\Delta 1-2-3$  that had introns 2 and 3 deleted, and pUCHs $\pi$ cDNA that had introns 1, 2, and 3 deleted (D. A. O'Brochta & A. M. Handler, unpublished results; plasmids provided by D. Rio, Whitehead Inst.). The *hs* $\pi\Delta 1-2-3$  helper worked well in *D. melanogaster*, but not in *Anastrepha*, though curiously, the *hs* $\pi$ cDNA did not function in either insect. The former result could be due to a lack of intron 1 splicing in *Anastrepha* consistent with the splicing assay results, but the latter result might indicate either a mutation in the cDNA plasmid construct, or a function for intron 1 in stable transcript biogenesis. We subsequently sequenced the first and second exons of the cDNA and found a single base deletion in exon 1, which is expected to cause a frameshift resulting in a nonfunctional gene-product. Further evaluation of transposase cDNA activity in nondrosophilids would therefore require correction of the mutation or isolation of a new cDNA. If eliminating the need to process the transposase transcript relieves a major restriction on P function, then the

cDNA may provide a functional helper for P-mediated transformation in nondrosophilids.

In addition to nonfunctional gene-product formation, truncated transposase products may also act to repress normal transposase function. For example, an internally deleted P-element, KP, has been associated with repression of P-mediated dysgenesis (4). It has also been proposed that the somatic product of the complete transposase gene, a truncated 66-polypeptide resulting from the unspliced third intron, has repressor activity (97). Using the excision assay, we tested this hypothesis in the *D. melanogaster* embryonic soma by co-injecting a plasmid with the full length transposase gene, *phs $\pi$* , along with the indicator and helper plasmids. Repressor activity was evidenced by a dose-dependent elimination of P excision with relatively low concentrations of *phs $\pi$*  (D. A. O'Brochta, S. P. Gomez, & A. M. Handler, unpublished results). If processing was merely inefficient in nondrosophilids, enough repressor could be produced to inhibit any normal activity. Conceivably, a transposase cDNA would alleviate transposase-encoded repressor formation, which could be verified by an immunological analysis of transposase gene-product formed in nondrosophilid embryos.

If a cDNA is functional in *Drosophila* but fails to function in nondrosophilids, and no transposase-encoded repressor formation occurs, essential cofactors are probably missing and/or non-P-encoded repressors exist in these species. The participation of transposase-independent factors in P mobility has been inferred by our analysis of excision products, and more directly by the discovery of a protein that has binding specificity for the P-terminal sequences (98). A general approach to evaluating these possibilities is to isolate putative positive-acting factors from *Drosophila* (as DNA, RNA, or protein) and test them by co-injection in mobility assays in nondrosophilids. Conversely, putative repressors may be isolated in nondrosophilids and their negative influence tested in mobility assays in *D. melanogaster*.

### *New Gene Vectors*

While the defect restricting P-element mobility in nondrosophilids might be corrected, leading to a functional transformation system, uncertainties still remain. For example, a negative factor may be identified but not easily eliminated, and while we may overcome restrictions in families closely related to Drosophilidae, additional restrictions may occur in families more distantly related. Thus, to achieve gene transfer in a broad range of insects, researchers will probably need to analyze other less restricted insect and noninsect transposon systems or develop methods to rapidly isolate and test species-specific transposon systems for development into gene vectors.

**OTHER TRANSPOSON SYSTEMS** Transposable elements appear to be ubiquitous components of eukaryotic genomes and, as with the P system,

several have been modified into gene vectors for the species in which they were discovered (24, 50). Transposable elements that are potential candidates for gene vector development should have unrestricted (or less restricted) mobility properties as indicated by an absence of tissue specificity, a broad phylogenetic distribution, or demonstrated mobility in a wide range of species. Three elements that meet these criteria are *mariner* from *D. mauritiana* (43), *Ac* from maize (29), and *Tc1* from nematodes (84).

The *mariner* element resembles P-elements structurally (51), but unlike P, *mariner* is normally active in both the germline and soma (45). Under appropriate conditions, it has a rate of mobility greater than that of P (11). Like P, *mariner* can be *trans*-mobilized by autonomous *mariner* elements (78), indicating that a binary vector-helper system, important to regulating the movement of a gene vector, may be feasible.

The *Activator/Dissociator (Ac/Ds)* transposon system from maize has thus far demonstrated the most phylogenetically unrestricted mobility properties of any known eukaryotic transposon. *Ac* elements have shown mobility, after *Agrobacterium* Ti plasmid-mediated transformation, in tobacco (1), tomatoes (120), carrots (61), and *Arabidopsis* (115). These observations indicate that either *Ac* is truly autonomous or non-*Ac*-encoded functions are highly conserved. Recent isolation of the *Ac* cDNA (44) makes possible the expression of the *Ac* transposase in animal cells. The *Tc* elements from *Caenorhabditis elegans* have also been well characterized and, like *mariner*, can be highly mobile in somatic and germline tissue (26). Interestingly, *Tc1* has a high degree of sequence similarity to the *HB* transposon family in *D. melanogaster* (46); the *Tc1* and *HB* open reading frames share about 30% amino acid homology. This homology indicates that *Tc* elements have had a long evolutionary history and/or were transmitted horizontally to insects. Although an autonomous *Tc* element has not been isolated, several candidates have been genetically identified (85). Testing *Tc1* mobility in insects will depend upon the isolation of these elements, which should be forthcoming.

A general strategy for initially testing these elements would follow the total excision assay for P. Excision reporter plasmids might consist of transposable element terminal sequences flanking a streptomycin-sensitivity gene insertion. Helper plasmids would consist of the element-specific transposase under *hsp70*-promoter transcriptional control. Mobility would be assayed after plasmid incubation in host embryos and subsequent testing for streptomycin resistance in transformed bacteria.

**NEW TRANSPOSON SYSTEMS** Transposable elements have been found in a wide range of prokaryotes (58) and eukaryotes (100) and are known to comprise a significant portion of the *D. melanogaster* genome (101, 110). They have often been identified by virtue of their mutagenic properties (39), and mutations resulting from the insertion of transposable elements often

display characteristics such as a high degree of instability. Recently, insect transposon-induced mutations were recovered in baculoviruses that had infected lepidopteran cell lines (33). In these cases, the viral genome acted as a target for transposon insertion. The ability to inject, transiently maintain, and recover bacterial plasmids from insect embryos may allow the similar capture of mobile genetic elements from host insects. In a system converse to the excision assay, plasmids injected into insect embryos could harbor target sequences that would facilitate the identification of insertion events, some of which should be transposable elements. Isolating transposons by virtue of their mobility properties would expedite their subsequent analysis and development into useful gene vectors.

**SITE-SPECIFIC RECOMBINATION** An alternative approach to achieving efficient gene transfer is to utilize site-specific recombination systems such as the *FLP* recombinase system of yeast (79), in which a recombinase protein facilitates specific recombination between *FRT* sequences. Insect target site strains might be created by having an *FRT* target site integrated into a host genome, possibly by random integration or inefficient gene transfer. Embryos from this strain would be co-injected with plasmids encoding the recombinase and marker genes with adjacent *FRT* sequences, which might then integrate by recombination with the chromosomal *FRT* sequences. The *FLP* recombinase system functions in *D. melanogaster* (36), and one can test its ability to promote recombination between plasmids in nondrosophilids. If successful, formidable efforts would be worthwhile to integrate *FRT* sequences into insect genomes to test this method.

### *Evaluation of Transformation Methodology and Selection*

**DNA INJECTION** The basic method for gene transformation in *Drosophila* has worked well, yet modifications will be necessary for other insects because of differences in their biology. For example, *Drosophila* eggs are injected soon after oviposition so that DNA may be introduced before cellularization and will be enveloped in the pole cells. This process occurs within 2 h of oviposition and most eggs are usually injected just before blastoderm formation. Insects with slower development have a greater preblastoderm period in which to inject, but this does not ensure that any time before cellularization is optimal. When a lepidopteran, *Plodia interpunctella*, was injected with plasmid shortly after oviposition, most of the DNA was degraded within 12 h and cellularization occurred at about 16 h (P. Shirk, personal communication). Though the cause of degradation is unknown, persistence of the injected DNA is necessary, which may need to be tested by injecting DNA at various times and assaying the transient somatic expression of a reporter gene, or determining relative rates of plasmid recovery.

Direct microinjection of preblastoderm insect eggs has been the common means of introducing DNA into germ cells. In *Drosophila*, and other species that have eggs with a relatively thin chorion that can be easily removed, this process is straightforward and survival rates can be quite high. For many other insects, this method is more challenging as a result of rigid or nonremovable chorions and sensitivity to desiccation resulting in low levels of DNA transfer or poor survival. In lepidopterans, researchers have been able to inject newly oviposited eggs that have a soft chorion (that later hardens) and seal the puncture with glue (107). Although imaginative approaches may eventually allow egg injection in most insects, the need to individually inject eggs, even for efficient transformation systems, remains a limiting factor. More universal methods allowing simultaneous DNA transfer into large numbers of eggs would certainly be preferable. Such methods would have the added benefit of allowing the testing, and possible implementation, of random DNA integration or inefficient gene vector systems.

**OTHER DNA DELIVERY SYSTEMS** Several systems for introducing DNA en masse into an organism's germ cells have been successful, and some are in the preliminary stage of testing in insects. One of the first involves shooting microprojectiles coated with DNA into target cells through a shotgun type mechanism (now commercially available from DuPont). Success has been achieved in *Allium cepa* epidermis (59), yeast mitochondria (52), and *Chlamydomonas* chloroplasts (7). Initial tests in insects indicate that DNA can be delivered into eggs this way, though the mortality rate is high and transformation has yet to be demonstrated (12). Mechanical introduction of DNA into cells has also been achieved by vortexing yeast in a DNA solution with glass beads (16). Similarly, DNA has been introduced into insect eggs by vortexing them in a DNA solution with silicon carbide fibers (A. Cockburn, personal communication). A recent report of the creation of transgenic mice using sperm encoated with DNA (67) has not been repeated by others (9), though some evidence supports DNA transfer with a similar method in honey bees, which can be artificially inseminated (81).

Several procedures are also used to mediate DNA uptake into cultured cells, some of which may be modified to allow uptake into insect oocytes. For example, electroporation has been used to permeabilize tissue culture cells of various prokaryotes and eukaryotes for transfection (106). Although pulses of high voltage may result in high mortality of insect eggs, this may not be limiting considering the ability to simultaneously treat large numbers of eggs. Less apparent damage to oocytes may be achieved by the use of calcium-phosphate precipitated DNA or DNA encapsulated in liposomes. Both these DNA treatments have resulted in transfection of mammalian and insect cell lines, as well as cellular uptake in vertebrates in vivo after intraperitoneal or

intravenous injection (3, 22). An analogous procedure in female insects would be the simple injection of DNA into the abdominal hemocoel, which, in *Drosophila*, has resulted in plasmid recovery from oviposited eggs (A. M. Handler & S. P. Gomez, unpublished results). Other methods may take advantage of receptor-mediated uptake where DNA is conjugated to molecules that are selectively sequestered by oocytes or cell nuclei (119). Selective uptake of DNA in liver cell nuclei has been achieved after conjugation to nonhistone chromosomal proteins and liposome fusion (53, 89). A similar approach in insects may utilize conjugation to histones, which after oocyte injection in *D. melanogaster* are selectively associated with nuclei (82), or conjugation to yolk proteins, which are selectively sequestered by oocytes (63).

Prolonging the presence of exogenous DNA in insect cells, especially through successive rounds of cell division and DNA replication, may enhance its ability to integrate either by a vector or through random integration. Extending the presence of DNA might be accomplished by linking the exogenous DNA to eukaryotic origins of replication or autonomously replicating sequences (ARS), which cause the persistence of episomal DNA. ARSs have been primarily isolated from yeast (112, 113), but similar sequences have been found in other organisms, including *Drosophila*. Interestingly, these nonyeast elements have ARS activity in yeast but lack this activity in their own cells (38, 74). Eliminating this restriction might enhance germline transformation and would almost certainly enhance transient expression analyses that depend upon persistence of exogenous DNA in somatic tissue.

**SELECTION FOR TRANSFORMANTS** The identification of putative transformants depends upon the ability to accurately select for chromosomal integration of the vector. This is relatively straightforward in *Drosophila*, in which a variety of genes resulting in a selectable phenotype can be linked to the DNA to be integrated. For example, the wild-type *rosy* gene confers a wild-type red eye phenotype when integrated into a *rosy* mutant genome (102, 103). Similarly, the wild-type *alcohol dehydrogenase* (*adh*) gene confers the ability to metabolize ethanol when integrated into *adh*<sup>-</sup> mutant hosts (35). Whereas *rosy*<sup>+</sup> transformants must be selected individually, the *adh* selection allows mass selection by ethanol resistance. Both of these selections, among others, are possible in *D. melanogaster* because mutant strains exist for which the wild-type gene is available as cloned DNA. In most nondrosophilid insects such mutant rescue selection is not yet possible, and so selectable marker genes must confer a dominant-acting phenotype. This may be in the form of chemical resistance or expression of a new visible phenotype. As discussed, a dominant-selection scheme has been developed for *Drosophila* using the bacterial NPT gene to confer resistance to neomycin or chemical analogs such as G418 (111). Since the NPT gene was purported to function in a wide range

of eukaryotic cells, this selection raised hopes that the P gene vector could be efficiently tested in various insects. NPT selection, however, has been inconsistent in its effectiveness.

Other genes confer dominant-selection phenotypes in cell lines but their usefulness in whole animals is unknown. The hygromycin-resistance gene has functioned in various eukaryotic systems including mammalian (71) and mosquito (J. Carlson & B. Beatty, personal communication) cell lines. Insect expression of dihydrofolate reductase may allow resistance to methotrexate (49), while the mammalian multiple drug resistance gene may allow resistance to various plant alkaloids (41). Although these resistance systems hold some promise, their use as selective agents may prove to be as problematic as neomycin resistance. Insecticide resistance genes may function more consistently as selective agents (87), though their practical use in transformation for field applications may be restricted.

Genes that confer a new visible selectable phenotype would have to be individually screened, but might be less prone to the inconsistencies of chemical selection. The bacterial *lacZ* gene has been transformed into *Drosophila*, resulting in high levels of  $\beta$ -galactosidase that yield blue coloration after a simple staining process (68). Although whole body staining would be lethal, body structures not critical to reproduction such as halteres, legs, or antennae can be dissected and stained allowing putative transformants to be mated. Similarly, the dominant expression of other detectable enzymes can be used for selection, such as chloramphenicol phosphotransferase (23) and luciferase (93). Direct screening for gene integration by DNA hybridization is feasible for efficient transformation systems (8), but this presents difficulties for insects that mate en masse, precluding the testing of siblings from established lines. This problem may be overcome by the use of polymerase chain reaction to detect integrated genes in superfluous tissues as described above. For most selections that require intermediate procedures for detection, it would probably be expedient to initially test groups of insects and subsequently focus on individuals.

Although limited, the genetic analysis of nondrosophilids has resulted in the recovery of some mutations that confer phenotypes similar to those found in *Drosophila*. Some of these may have a related genotype as well as can be complemented by cloned *Drosophila* genes. For example, the *topaz* gene in the biosynthetic pathway leading to the brown ommochrome eye pigment in *Lucilia cuprina* is homologous to the *scarlet* ommochrome gene in *D. melanogaster* (25). Prior to using these genes as germline transformation mutant rescue markers, functional complementation may be determined by transient expression experiments. For example, preliminary results indicate that transient expression of the *vermillion* gene in *Drosophila* can, at least partially, complement the *yellowish* eye mutation in *Lucilia* (P. Atkinson, personal communication).

Although selection in some insects may take advantage of gene homologies with *Drosophila*, the need for selection in a broad range of insects will depend upon more generally applicable methods. In lieu of mutant rescue, a converse selection by induction of a mutant phenotype may be eventually feasible by using ribozymes or DNA encoding antisense RNA for conserved genes having a selectable phenotype. This system would work, simply, by vector-encoded antisense RNA inhibiting the normal translation (or possibly transcription) of the complementary sense RNA from a normal resident gene, resulting in a loss of gene-product (see 40, 114a). In *Drosophila*, only mutant phenotypes visible in embryos or larvae have been induced in this way, and general inconsistencies in the activity of antisense RNA make this method prospective. Nevertheless, the potential broadbased utility of antisense RNA or ribozymes for selection should encourage their testing.

## CONCLUDING REMARKS

The need to achieve efficient methods for germline transformation in economically important insects remains a high priority. Although other methods exist to analyze the expression of manipulated insect genes, none of them are optimal for critical analyses nor are they useful for stable gene integration required for biological control methods. The *Drosophila* P-element gene vector system has not facilitated gene transfer in nondrosophilids, yet limitations and inconsistencies in DNA delivery and gene-integration selection systems have impeded a fair evaluation of the P vector, at least based on germline transformation experiments. Clearly an evaluation of P, or any other gene vector or transformation methodology, will first require the development of effective and reliable ancillary methods. Nonetheless, more direct embryonic mobility assays also indicate a lack of P function in nondrosophilids, and modifications of the P-vector system will be essential. These mobility assay methods, however, also allow the testing of P modifications to possibly define and ameliorate restrictions. While the restrictions may be overcome in some insect families closely related to drosophilids, more distantly related species may have other restrictions. Similarly, the development of other gene-transfer methods may not be universal, and transformation for a broad range of insects may ultimately depend upon methods allowing the efficient isolation and testing of new gene vectors for specific groups of insects.

## ACKNOWLEDGMENTS

Appreciation is extended to those who shared their unpublished results with us, to Drs. P. Greany, A. Malavasi, S. Miller, and H. Oberlander for comments on the manuscript, and to the USDA-Cooperative State Research Service for support.

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