

AN ASSESSMENT OF GENE TRANSFORMATION IN INSECTS

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Introduction

The manipulation and transfer of genes into various insect species offers a vast potential for increasing our ability to study the genetics, biochemistry, and development of these species, as it has done already for Drosophila melanogaster. For those insects that are economically important, gene manipulation coupled with an efficient transformation system would greatly enhance insect management programs by providing, for example: 1) a means for developing efficient genetic sexing and sterilization schemes for sterile male release programs; 2) a means by which insecticide resistance might be conferred to beneficial insects such as honeybees and hymenopterous parasites, 3) a means for conferring cold-hardiness or freeze tolerance to insects permitting storage of insects used in mass release programs, and 4) allowing introduction of genetic markers into populations for the purposes of monitoring gene flow, insect migration, and dispersal patterns.

More basic scientific information to be derived from genetic engineering would, in addition, lead to a more complete understanding of insect biology relevant to insect management and control such as resistance mechanisms, sex determination, hybrid sterility, and hormone action and metabolism (1,2).

Despite the benefits to be derived from gene-transfer methods in insects, the routine and efficient introduction of exogenous DNA into a host insect's genome is limited to the genus Drosophila (3). The P-element transposons from Drosophila are the only vectors available for whole insect transformation (3,4). Their functionality in other species, however, has not been fully assessed. Although species-specific gene transformation is not yet possible in non-drosophilid insects, genes can be isolated as cloned recombinant DNA from these species and introduced into the D. melanogaster genome by transformation. For

some genes, this may allow a dissection of the components regulating gene expression during development. Mitsialis and Kafatos (5) have transformed Drosophila with the chorion A and B genes from the distantly related species Bombyx mori. These genes were expressed in Drosophila with normal sex, tissue, and temporal specificities suggesting that further analysis of cis- and trans-acting regulatory components may be studied in Drosophila transformants. On the other hand, Wyatt and co-workers (6) transformed Drosophila with the Locusta migratoria vitellogenin B gene (having a partial internal deletion) and failed to observe gene expression. In lieu of the deleted region having a required function or other technical problems it appears that the 5' regulatory sequences, or regulatory molecules, of the Locusta vitellogenin gene have become divergent enough to prohibit their expression in Drosophila. Information may be gained by further analysis that may reveal the regulatory block(s), but clearly, a straightforward analysis of locust vitellogenin gene expression is not possible in Drosophila. Even with the more promising situation with Bombyx chorion genes, the elucidation of regulatory properties as a function of expression in Drosophila must be considered in the context of a foreign cellular environment.

It is worthwhile noting that some of the advantages of genetic analysis after transformation may be achieved more simply by transient expression analysis, and this technique is probably achievable in most insects. Martin et al. (7) found that when plasmids containing the alcohol dehydrogenase gene were injected into Drosophila embryos, these plasmids were transiently maintained as extrachromosomal DNA in the developing insect and expression could be detected as late as the third larval instar. In this way the developmental regulation of gene expression could be assessed independent of stable germline transformation. The clear advantage of transient expression is that gene expression can be analyzed in the same species, in the correct cellular environment. A drawback of this technique is that it is impossible to control the amount or distribution of functional, transiently maintained DNA in insect embryos or larvae, making quantitative analyses difficult. For particular genes in most insects, transient expression analysis does provide a relatively simple means of asking some important questions. As discussed further on, we have successfully utilized transient expression of P-element DNA to determine its functionality in drosophilids and tephritids. Heterologous transformation and transient expression techniques do provide some avenues of molecular genetic analysis for non-drosophilid insects, although clearly these techniques are not optimal, and do not appear useful for applied purposes such as insect management programs. The development of species-specific or a phylogenetically unrestricted transformation system therefore remains a high priority.

P-Element Mediated Gene Transfer in Drosophila

P-elements are highly mobile transposable elements initially isolated from

D. melanogaster and subsequently found in a number of species within the genus (8,9,10). P-elements have been modified structurally such that foreign DNA can be incorporated without destroying their ability to be mobilized (3). Following introduction of P-element vector DNA into embryonic germ cells by direct injection into preblastoderm embryos, up to 40-50% of the resulting fertile adults will produce at least one transformed offspring (4). P-elements also have been used as gene vectors in the sibling species D. simulans (11), and P transposition has been demonstrated in the more distantly related species D. hawaiiensis (12). These results suggested that P mobility might be phylogenetically unrestricted and that P might be useful as a gene vector in a wide range of species.

The development of P vectors carrying the neomycin phosphotransferase (neo) gene allowed neomycin resistance to be used as a means of selecting transformed individuals (13), which has been especially useful for introducing DNA sequences into species or strains for which there is no selection. However, the use of P vectors carrying the neo resistance gene to assess P functionality in heterologous systems suffer from two limitations. First, P mobility is not being tested directly. It cannot be assumed that P-elements carrying exogenous DNA will have the same mobility properties as unmodified elements. The efficiency with which transformants can be recovered in D. melanogaster is related in some cases to the amount of exogenous DNA the P-element contains (4). How or if this size constraint will change in heterologous systems is unknown. Brennan et al. (12), while successful in demonstrating mobility of autonomous, unaltered P-elements in D. hawaiiensis, were unable to detect mobility of a P vector carrying exogenous DNA. Second, detecting the mobilization of a P-element carrying a dominant selectable gene construct (neo resistance) relies on the adequate expression of the construct so the selectable phenotype is conferred (e.g., whole animal resistance), which cannot be assumed. Therefore, unless use of P vectors carrying dominant selectable markers yield a transformed animal, little information regarding the mobility of P-elements or their utility as gene vectors in heterologous systems is gained.

P-element mediated transformation of a non-drosophilid has not been reported thus far. Recently a transformation study using the P-element system in Anopheles gambiae recovered one integration event following the injection of 2,279 embryos (of which 24% survived) (14). Upon analysis it was concluded that the integration event did not result from P-element transformation. Efforts by our laboratory and others have failed thus far to recover a P-element mediated integration event in tephritids. In the medfly, Ceratitis capitata, transformation studies by several labs involving injection of many thousands of embryos have failed to yield a genetic transformant (15). This indicates that in tephritids either i) P is not mobile, ii) whole animal neo resistance cannot be conferred with the gene construct used, iii) P vectors suffer from stringent size

or structural constraints, or iv) P target sequences are unavailable in the host genome.

P-element sequences have also been shown to increase the transfection frequency of mammalian cells (16). However, as with *Anopheles gambiae*, these integration events were not the result of P transposition and do not indicate P functionality in this system.

Therefore, to assess and analyze the functionality of P-elements in non-drosophilids, methods are required that allow assessment of i) P mobility directly, ii) P vector mobility independent of the dominant selectable marker it carries, and iii) P target site availability in the host genome. These methods must be rapid, simple, and allow modifications of P-elements and P vectors to be tested.

Evaluating P-Element Mobility in Non-Drosophilids

P-element insertions often result in partial or complete gene inactivation, and reversion of these insertional mutations usually result from the precise or nearly precise excision of the transposon. In *D. melanogaster* P-element excision is biochemically related to transposition because it requires the P-encoded polypeptide transposase and an undefined cellular condition known as M cytotype (17). Because P-element excision is related to transposition, it provides a way of monitoring the functionality of the P-element transposon system.

Rio et al. (18) recently took advantage of these characteristics of P movement, and developed 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 non-autonomous P element sequence surrounded by *D. melanogaster white* gene DNA was inserted into the lacZ alpha peptide coding region of pUC8. In this configuration, lacZ, which is required in appropriate bacterial hosts for beta-galactosidase activity, is nonfunctional. When the P-element excises, lacZ regains function, and beta-galactosidase activity can be detected by blue coloration of bacterial colonies that have been transformed with the plasmid and cultured on X-gal containing media.

We have modified the excision assay so that P functionality may be assessed in the insect embryonic soma enabling us to address directly the question of P mobility in non-drosophilids (19). The pISP indicator plasmid constructed by Rio et al. (18) was co-injected with a helper plasmid (pUCHs Δ 2-3), providing a source of transposase, into preblastoderm insect embryos. After the plasmids were transiently maintained in embryos for 16-24 hr, P-element excision was assayed as a function of lacZ activity. This assay tests the ability of the transposase gene to be processed into a functional gene-product, as well as determining more generally whether the embryonic milieu is supportive of P

mobility. After performing the excision assay in a variety of drosophilids, and two tephritid species, we found that P-elements can be efficiently mobilized in drosophilids, including representatives outside of the Drosophila genus, but that P mobilization did not occur in the distantly related Tephritidae. This suggests to us that current P-element-based gene vectors may not function in other insect systems. Significantly, excision assay results from D. melanogaster indicate that functional transposase in the embryonic soma can support a level of P mobility comparable to that observed in germline tissue (8). This should allow us to predict directly the frequency of P-element transformation from somatic excision assay results. For example, germline transformation in D. melanogaster occurs at a frequency of $2-4 \times 10^{-1}$ /surviving embryo; and we have measured somatic excision assay events at a frequency of 1.7×10^{-3} /pISP indicator plasmid. If, for example, a minimum germline transformation frequency of 5×10^{-3} were desired in another species, then a somatic excision assay frequency of 10^{-5} /indicator plasmid would be required. This would indicate to us that the P-element vector system (or modification of the system) and the embryonic environment could support a minimal level of P mobility necessary for germline transformation.

Interpretation of P-Element Excision Results

Interpreting results of the transient excision assay requires consideration of the following points. First, the relationship between P excision and insertion is not fully understood. While P transposition does have a replicative component, there also exists a strong correlation between the occurrence of P-element excision and transposition suggesting that excision is a step in the transposition process. Despite the uncertainty of the mechanism of transposition it is clear that conditions required for these two events appear linked indicating a shared biochemistry. Second, only precise or nearly precise excisions can be detected using this assay. Excision events that do not restore lacZ alpha peptide activity will remain undetected. The frequency of precise excision in D. melanogaster varies from locus to locus, and where measured the frequency of imprecise excision can be as high as 75% (9). Although the significance of these results is hard to assess within the context of the excision assay used in these experiments, they suggest that the precise excision events measured may be an underrepresentation of all excision events, and therefore, an underestimation of P-element functionality. This must be considered when interpreting negative results or low excision frequencies. Third, a question remains as to whether somatic excision accurately reflects P-element behavior in the germline. This is especially significant if somatic excision is to be used to determine conditions necessary for germline transposition. That somatic excision events do reflect germline behavior is supported by the observation of P-element excision and germline transposition in the Drosophila species melanogaster and simulans. In addition, germline transposition occurs in D. hawaiiensis (12), and we report somatic excision in

the closely related species *D. grimshawi*. These results suggest that somatic excision is a reliable predictor of P-element behavior in the germline.

Determining the Basis of P Dysfunction in Non-drosophilids.

Our current understanding of P-elements and the properties regulating their movement provide us with a basis from which modifications can be developed to potentially overcome mobility restrictions in non-drosophilid species. The ability of an autonomous 2.9 kb P-element to transpose depends upon the integrity of its 31 base pair terminal inverted repeats (20,18) and an internal transcription unit composed of four open reading frames encoding an 87 kDa protein required in transposition (21,18). This protein, or "transposase," is required for both excision and transposition, although its precise mechanistic role is unknown. There are at least two levels of control regulating P-element movement with one of these directly affecting transposase function. First, P-elements can only be mobilized when present in a rather poorly defined cellular state known as M cytotype (17,22). This maternally inherited cellular property is usually found in strains lacking autonomous P-elements. Strains containing autonomous P-elements usually develop a cytotype (P) which represses P movement (22). The maternally inherited cellular factors that determine cytotype are unknown, however, a recent report (23) indicates that truncated, non-functional transposase polypeptides may act as repressors. Second, P-element movement is limited to the germline (17,24). Although P-element transcription occurs in all cells, complete transcript processing required for functional transposase is germline specific (25). It has been observed that removal of intron 3 and splicing of exons 2 and 3 occurs only in the germline and is apparently the basis for the tissue specificity of excision and transposition (25). 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 *Drosophila* somatic tissue.

While splicing of introns 1 and 2 occurs normally in *Drosophila* somatic cells, it is unknown whether splicing of any, or all introns occurs in non-drosophilids. We have demonstrated that transposase transcription occurs from P-element containing plasmids transiently maintained in *A. suspensa* embryos (19), although it has yet to be determined whether complete intron splicing occurs. This may be determined by mapping transcripts in RNAase protection experiments, or by determining whether transposase transcription units lacking all introns (i.e., cDNA) can result in functional transposase in the excision assay. The failure of P-element constructs lacking introns 1, 2 and 3 to mobilize P-elements in non-drosophilid embryos might indicate that either positive acting co-factors are lacking in these embryos, or negative acting factors restricting mobility are present. Non-P encoded functions have recently been implicated in the regulation of P-element transposition (26), and such functions have been suggested for plant

and bacterial transposon systems (27,28).

Positive-acting factors may be discovered by isolating either nuclear extract fractions, crude ooplasm, total RNA, and/or polyA⁺ RNA from Drosophila M strain oocytes and testing their ability to facilitate P mobility in an excision assay in non-drosophilid embryos. Negative factors could be tested in a converse analysis, by isolating similar molecules from non-drosophilid or P strain oocytes and determining whether they act to inhibit P mobility in Drosophila embryo excision assays. If transposase transcripts are processed normally, but not translated, then transposase produced in an expression system may be tested. That such co-injection experiments can succeed is supported by studies in Drosophila which report complete or partial rescue of mutant phenotypes after injection of mutant embryos with RNA or ooplasm from wild type embryos (29,30). Rescued mutant phenotypes have ranged from embryonic pattern formation defects (30) to adult phenotypes related to pyrimidine metabolism (31).

While in this way the defect restricting P-element mobility in non-drosophilids may be corrected leading to a functional transformation system, uncertainties still remain. For example, a negative factor may be identified but not easily eliminated. In this case it would be necessary to develop a species-specific transformation system analogous to the P-element system.

Development of a Transformation Gene-Vector in Non-Drosophilids

The development of a germline transformation system which is not dependent upon the D. melanogaster P-element, will, nevertheless, probably depend upon the isolation and structural modification of transposable elements. Several transposable elements, other than P, have been identified in Drosophila and some hold potential for being developed into new gene vectors. For example, copia is a retrovirus-like element found throughout the family Drosophila and is apparently of ancient origin (33,34). Although copia is widely distributed phylogenetically, it has a very low frequency of transposition in laboratory strains. This, however, is apparently not the case in insect cell lines, or when copia containing strains are placed under environmental stress (35,36,37). As more is learned about the control of copia transposition, its utility as a gene vector will become more apparent.

Of more immediate use might be the recently isolated transposable element hobo from D. melanogaster (38,39). Hobo elements are 3.0 kb in size, contain a 1.9 kb open reading frame, and in several respects closely resembles the Drosophila P-element. The existence of apparent deletion derivatives of full length hobo elements, that appear to be mobile in response to trans-activity from autonomus elements, suggests that hobo may be amenable to similar structural modification as made to P, thereby facilitating their development into gene vectors.

Recently a transposable element (mariner), which acts in both the soma and

the germline of *D. mauritiana*, was isolated (40). Although not much is known about the structural and biochemical requirements for *mariner* transposition, its unrestricted mobility within *D. mauritiana* suggests that it might be useful as a gene vector in heterologous systems.

The potential phylogenetic restricted mobility of transposable elements may necessitate the isolation of endogenous elements from the insect of choice. That transposable elements do exist in non-drosophilids is indicated by the identification of transposons in *Bombyx* (41,42), *Trichoplusia ni* (43,44) and *Ephesia* (45). The identification and isolation of additional transposable elements might be accomplished in a number of ways. For example, libraries of mobile repetitive DNA constructed from isolated populations of a species might be compared. Sequences unique to one population might represent unstable, mobile DNA sequences. Similar analyses could also be conducted between closely related species (46).

Retrovirus-like transposable elements are thought to transpose via RNA intermediates. In *Drosophila* cell lines, virus-like particles containing RNA sequences homologous to the retrovirus-like transposable element *copia* , have been detected as well as reverse-transcriptase-like activity (47,48). Analysis of virus-like particles and extrachromosomal circular DNA from other insects and insect cell lines might lead to the identification of transposable elements that could be developed into gene vectors.

The phenomenon of hybrid dysgenesis has led to the discovery of I mobile elements (32), as well as P-elements, in *D. melanogaster*. Non-reciprocal gonadal dysgenesis has also been reported in *Chironomus thummi* hybrids (49). The discovery and analysis of non-reciprocal hybrid dysgenesis in non-drosophilids may also lead to the isolation of useful transposable elements. Transposable elements have been isolated by virtue of their mutagenic effects within the host genome and more recently within the genome of viruses that are transiently maintained in insect cell lines (44). The transient maintenance of transposable element target sequences (e.g., viral genomes) in insect cells or cell lines might provide a unique way of capturing mobile genetic elements of host origin. This method would not rely on the isolation of host mutants and would result in the isolation of elements that have demonstrated mobility.

Summary

The full exploitation of molecular biological analyses in non-drosophilid insects, for both basic and applied purposes, will depend upon the ability to assess the activity of recombinant DNA molecules during development of the insect. Particular genes from certain insects may be amenable to transformation analysis in *Drosophila melanogaster*, or transient expression analysis within the same species. Nevertheless, limitations of these techniques make the development of a phylogenetically unrestricted germline transformation system necessary and

of primary importance. We have developed an *in vivo* excision assay which has allowed us to evaluate the ability of the *D. melanogaster* P-element to function in non-drosophilid insects. While P function has not been detected in tephritids, using the excision assay, modifications of the P-element based vector system can be tested quickly and quantitatively. If the P-element system is not amenable to modification, efficient gene transformation in insects will depend upon species-specific transformation systems. These systems will most likely be based upon transposable elements analogous to the P transposon. Some transposons have already been isolated from insects, and techniques are available to isolate and evaluate others.

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