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Genetics

Mobility of *P* elements in drosophilids and nondrosophilids

(transposons/excision assay/Drosophilidae/Tephritidae)

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ABSTRACT **The mobility properties of the *Drosophila melanogaster P* element in drosophilid and nondrosophilid species has been determined using a *P*-element mobility assay that is conducted transiently in insect embryos. *P* elements are mobilizable in all drosophilids tested, including species outside the genus *Drosophila* but not in the related Tephritidae (order: Diptera), although the *P*-element gene necessary for mobility, transposase, is transcribed. These results show that without modifications *P* elements will not serve as general insect gene vectors and suggest that nonconserved host-encoded factors participate in the transposition of *P* elements. Our methods will be generally useful for analyzing the cis- and trans-acting factors required for *P*-element mobility *in vivo* and could be used to analyze the mobility properties of other transposable elements in insects.**

P elements are highly mobile transposable elements originally isolated from *Drosophila melanogaster*, but also found in other species in the genus *Drosophila* (1–4). The mobility properties of this transposon allowed its development into an efficient gene vector, which significantly enhanced our ability to study all aspects of *Drosophila* biology (5, 6). The ability of *P* elements to be mobilized in the sibling species *Drosophila simulans* (7) and the more distantly related species *Drosophila hawaiiensis* (8) suggested that *P*-element movement is phylogenetically unrestricted, allowing its use as a general gene vector in a variety of insect and noninsect systems. This possibility was enhanced by the development of *P*-element vectors carrying the dominant selectable marker neomycin phosphotransferase, which potentially allowed testing of *P*-element transformation in heterologous systems (9). Nevertheless, the most straightforward transformation experiments would not measure *P*-element mobility directly and would rely on a selection system that is untested in most insects. Thus, negative results would not necessarily reflect a failure of *P* elements to be mobilized and would reveal few insights into the function of the *P*-element transposon system in nondrosophilids. Although a single gene-transformation event was reported for one nondrosophilid species, *Anopheles gambiae* (10), this event did not result from *P*-element transposition.

An initial step in assessing the utility of the *P* element as a general gene vector in insects is to directly determine the phylogenetic limits of *P*-element mobility. If limits do exist, understanding their basis will undoubtedly reveal information about the mechanisms and regulation of *P*-element transposition as well as an understanding of the origin and distribution of *P* elements within the genus. Determining the phylogenetic limits of *P*-element mobility will depend upon monitoring either *P*-element transposition and insertion or *P*-element excision. *P*-element insertions often cause partial or complete gene inactivation, and reversion of these insertional mutations usually results from the precise, or nearly precise, excision of the transposon (11, 12). In *D. melanogaster*, *P*-element excision is related to *P* transposition in that it requires the *P*-encoded polypeptide transposase and an M cytotype (6, 13). The mechanistic relationship between *P*-element excision and transposition is unknown, but because *P*-element excision is biochemically related to transposition, it provides a way of directly monitoring the function of the *P*-element transposon system.

Rio *et al.* showed that *P* elements can excise not only from resident sites in the genome, but also from plasmids introduced into insect (14) and mammalian cell (15) lines producing *P* transposase. Here we describe a modification of the *in vitro P*-element excision assay of Rio *et al.* (14) to directly assess *P*-element mobility in insect embryos. This assay can evaluate function of the *P*-element system in nondrosophilids and facilitates an analysis of mechanisms that regulate *P*-element mobility in *Drosophila*. We find that *P* elements can be efficiently mobilized in a variety of drosophilids, including representatives outside the genus *Drosophila*, but not in the related Tephritidae. These results are significant because they indicate that *P*-element mobility *in vivo* is phylogenetically restricted, limiting the utility of *P* elements as a general insect gene vector. Furthermore, these results suggest that nonconserved, host-encoded functions other than the *P*-element-encoded transposase are necessary for *P*-element mobility. This method should be generally applicable to the analysis of *P*-element function in other insects and to the identification and analysis of other insect transposable elements.

MATERIALS AND METHODS

Plasmids. The plasmids pISP and pISP-2 are *P*-element excision indicator plasmids constructed and used by Rio *et al.* (14) to assess *P* transposase activity in cell lines (Fig. 1). These plasmids consist of a 47-base pair (bp) fragment from the *D. melanogaster* white gene containing a *P*-element target sequence inserted into the polylinker region of pUC8 so as to retain the *lacZα*-encoded peptide-complementing function of that plasmid. Into the *P*-element target sequence was inserted a 600-bp nonautonomous *P* element resulting in an 8-bp duplication of the white sequences adjacent to *P* and a loss of LacZα complementation (14). The plasmids pISP and pISP-2 were used interchangeably and are referred to as pISP. pUChsπΔ2-3 has been described and consists of the 4.6-kilobase (kb) *Bam*HI fragment from pCNΔ2-3 containing the *P*-element transposase gene (open reading frames 0–3) lacking the third intron and under the promoter control of the *D. melanogaster* 70-kDa heat shock protein gene inserted into the *Bam*HI site of pUC8 (16). Insertion of the *Bam*HI fragment eliminates the LacZα-complementing function of this plasmid. The plasmid pACYCHhsπΔ2-3 was constructed by inserting the 4.6-kb *Bam*HI fragment containing hsπΔ2-3 from pUChsπΔ2-3 into the *Bam*HI site of pACYC184 (17), which does not contain the LacZα peptide-coding region or confer ampicillin resistance. Although both pUChsπΔ2-3 and pACYChsπΔ2-3 encode transposase, the *P*-element se-

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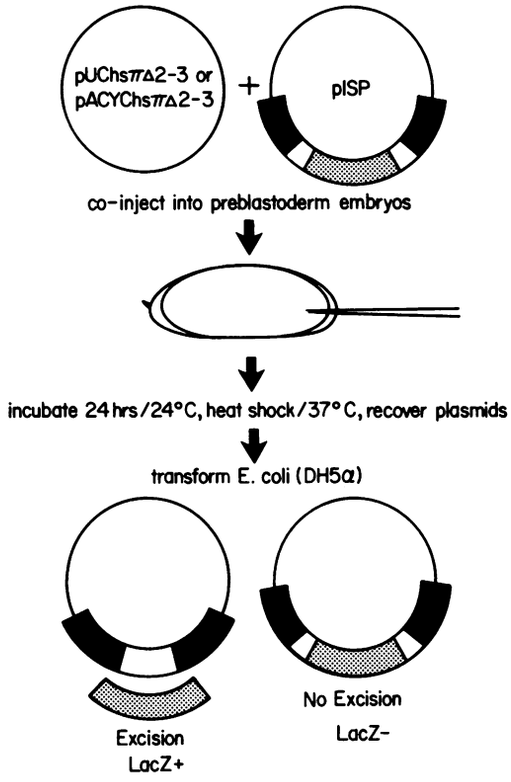

FIG. 1. *In vivo P*-element excision assay. Continuous line, pUC8 DNA containing an ampicillin-resistance gene; solid bar, LacZα peptide-coding region; open bar, *P*-element-insertion site from the white gene; stippled bar, nonautonomous *P*-element DNA. pUChsπΔ2-3 and pISP are pUC8 derivatives conferring ampicillin resistance and with an interrupted LacZα peptide-coding region. pACYChsπΔ2-3 confers chloramphenicol resistance and does not contain a LacZα peptide-coding region. The *P* sequences contained within the helper plasmids cannot be mobilized due to lack of complete terminal inverted repeats.

quences contained within these plasmids cannot excise or transpose because of the absence of complete terminal inverted repeats.

Excision Assay. *Microinjections.* The *P*-element-excision indicator plasmid (pISP) and the transposase-producing helper plasmids (pUChsπΔ2-3 or pACYChsπΔ2-3) were introduced into insect embryonic cells by direct injection into preblastoderm embryos using the methods developed for *D. melanogaster* (ref. 5; Fig. 1). Because most plasmids introduced into preblastoderm embryos are incorporated into somatic cells (18), which are unable to splice the third intron from the transposase transcript (15), helper plasmids containing a transposase gene lacking the third intron (hsπΔ2-3) were used. Removal of the third intron allows the production of fully processed transcripts and functional transposase in the soma of *D. melanogaster* (16). When pISP was coinjected with either pUChsπΔ2-3 or pACYChsπΔ2-3, each plasmid had a concentration of 0.5 mg/ml. In control experiments in which only pISP, pUChsπΔ2-3, or pACYChsπΔ2-3 was injected, a plasmid concentration of 1 mg/ml was used. We estimate that upon coinjection of pISP and the helper plasmid 10^7–10^8 pISP plasmids were introduced into each embryo. After injection embryos were incubated under O_2 at 24°C, heat shocked ≈18 hr after injection at 37°C for 1 hr, and allowed to recover at 24°C under O_2 for 1 hr before plasmid recovery.

Plasmid recovery. Low-molecular weight DNA was recovered from injected embryos as described by Hirt (19). As many as 100 embryos were extracted in 100 μl of Hirt lysis buffer (19). Low molecular weight DNA was resuspended in 6 mM Tris, pH 7.4/0.1 mM EDTA/10 mM NaCl and used to transform LacZ⁻ *Escherichia coli* hosts (DH5α).

Transformation. Ten embryo equivalents of low-molecular weight DNA were used to transform 200 μl of transformation-competent DH5α cells (originally obtained from Bethesda Research Laboratories) prepared according to the method of Hanahan (20). Transformants were plated on LB plates (21) containing ampicillin (75 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 50 μg/ml). Approximately 10^3 ampicillin-resistant transformants per embryo equivalent of low-molecular weight DNA were routinely recovered. Recovery of pISP plasmids from injected embryos and introduction of these plasmids into DH5α hosts permitted transformants containing pISP plasmids lacking the *P* element, as a result of precise or nearly precise excision, to be detected as LacZ⁺ (blue) colonies on X-gal-containing indicator plates. Restriction endonuclease digestion of putative excision products with the enzymes *Eco*RI and *Hin*dIII was used to confirm the loss of *P*-element sequences.

RNA Analysis. RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (22) and was size fractionated by electrophoresis in a 1% agarose–formaldehyde gel and transferred to nitrocellulose (23). Filters were hybridized in 50% (vol/vol) formamide at 50°C and washed in 0.25× SSC (1× SSC = 0.15 M sodium chloride/0.015 sodium citrate, pH 7), 0.1% $NaDodSO_4$ at 60°C. The probe was a uniformly labeled ([^{32}P]CTP, 800 Ci/mmol; 1 Ci = 37 GBq) 729-nucleotide single-stranded antisense RNA generated from a Gemini 2 vector (Promega Biotec, Madison, WI) containing the 689-bp *Xho* I–*Hin*dIII fragment from pUChsπΔ2-3 (24).

RESULTS

Using an *in vivo P*-element excision assay we measured the mobility of *P* elements in embryos of seven drosophilid and two tephritid species, including the Caribbean fruit fly *Anastrepha suspensa* and the papaya fruit fly *Toxotrepana curvicauda*. Table 1 shows that *P*-element excision from the pISP indicator plasmid occurred at a frequency ranging from 10^{-4} to 10^{-3} per pISP plasmid recovered from embryos of all drosophilids tested including the *D. melanogaster* P strain Harwich, *Chymomyza proncemis*, and *Zaprionis tuberculatus*. Restriction endonuclease digestion of LacZ⁺ pISP plasmids with *Hin*dIII and *Eco*RI confirmed the loss of *P* sequences (Fig. 2). The frequency of excision in the distantly related drosophilids *C. proncemis* and *Z. tuberculatus* was less by a factor of 10 than in the *D. melanogaster* M strain. Screening more than 10^5 plasmids from the tephritids *A. suspensa* and *T. curvicauda* failed to reveal evidence of *P*-element excision events (Table 1).

In control experiments in which only pISP or pUChsπΔ2-3 was injected, LacZ⁺ plasmids were occasionally recovered from some species. These LacZ⁺ plasmids were only recovered when plasmid DNA was maintained in insect embryos and were not recovered when purified plasmid DNA was used to transform DH5α cells (data not shown). Therefore, the LacZ⁺ plasmids were not rare contaminants of the original plasmid preparation, but more likely arose from recombination events. Although the frequency with which LacZ⁺ plasmids were recovered after injection of only pUChsπΔ2-3 was one per 10^3 plasmids, we find that this plasmid perhaps due to its larger size is not recovered as efficiently as pISP. In coinjection experiments using pISP and pUChsπΔ2-3, 10% of recovered plasmids were pUChsπΔ2-3. Therefore, during coinjection experiments LacZ⁺ pUChsπΔ2-3 plasmids were a small fraction of the total LacZ⁺ plasmids recovered. Because these putative spontaneous rearrangements were rare and inconsistently detected,

Table 1. Frequency of *P*-element excision from plasmids transiently maintained in insect embryos

Host insect	pISP (excision indicator)							
	pUChsπΔ2-3 (helper 1)		pACYChsπΔ2-3 (helper 2)		No helper		pUChsπΔ2-3 alone	
	Frequency* ($\times 10^{-3}$)	n ($\times 10^3$)	Frequency* ($\times 10^{-3}$)	n ($\times 10^3$)	Frequency* ($\times 10^{-3}$)	n ($\times 10^3$)	Frequency† ($\times 10^{-3}$)	n ($\times 10^3$)
Drosophilidae								
Genus *Drosophila*								
Subgenus Sophophora								
D. melanogaster M (*Adh*^{fn23} *cn*; *ry*^{506})	1.7	82	1.6	11	0	69	0.15	7
D. melanogaster P Harwich	0.5	82			0	22		
D. simulans	0.85	47	0.6	15	0	13	0	7
D. willistoni	0.92	3	1.0	2	0	12	0	5
Subgenus *Drosophila*								
D. melanica	0.52	14			0.05	18	0.19	5
D. grimshawi	0.6	10						
Genus *Chymomyza*								
C. proncemis	0.2	30			0	137	0	2
Genus *Zaprionus*								
Z. tuberculatus	0.2	28	0.3	3	0.03	35	0.24	8
Tephritidae								
Anastrepha suspensa	0	143						
Toxotrypana curvicauda	0	105						

*Frequency = LacZ$^+$ per pISP recovered; n = pISP recovered; when pUChsπΔ2-3 was used as a helper, we estimated n to be 0.9 × ampicillin-resistant colonies (data not shown).
†Frequency = LacZ$^+$ per pUChsπΔ2-3 recovered; n = pUChsπΔ2-3 recovered.

their occurrence did not significantly affect the observed excision frequencies from pISP derived from coinjection experiments. To avoid any possible ambiguity from recovery of LacZ$^+$ helper plasmids, we constructed a plasmid containing hsπΔ2-3 on the P15A replicon pACYC184, which does not contain a LacZα peptide-coding region or a β-lactamase gene (17). This permitted screening of only pISP plasmids after coinjection of pISP and pACYChsπΔ2-3. Table 1 shows that use of this new helper plasmid resulted in excision frequencies similar to those obtained using the helper pUChsπΔ2-3.

Although heat shock of *D. melanogaster* embryos was not required to detect *P*-element excision (data not shown), the transcriptional activity of the *hsp70* promoter in tephritids was undetermined. Therefore, RNA blot analysis was conducted on total RNA isolated from *A. suspensa* and *D. melanogaster* (M strain) embryos injected with the helper plasmid pUChsπΔ2-3. The results demonstrated the presence of *P* element-homologous RNA species 3.0, 1.5, and 1.0 kb in length in both *A. suspensa* and *D. melanogaster* (Fig. 3). These transcription products were not detected in uninjected embryos. In both species the 3.0-kb transcript was seen in all experiments; however, the occurrence of the 1.5- and 1.0-kb transcripts was variable. We also occasionally detected a 3.5-kb transcript in both *D. melanogaster* and *A. suspensa* embryos injected with helper plasmids. In comparison, the most prominent *P*-element transcripts in the P strain π2 are 2.5, 1.4, and 0.9 kb, whereas strains harboring a single copy of the *P*-element *Pc*[*ry*], from which hsπΔ2-3 was derived, contain *P*-element transcripts between 2.5 and 3.0 kb (25). The *P*-element transcription products seen in tephritids are therefore consistent with transcript sizes in *Drosophila* seen here and in previous studies. These results demonstrate that the *hsp70* promoter can function in tephri-

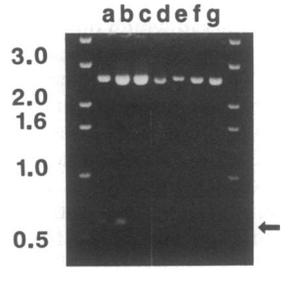

FIG. 2. Restriction endonuclease digestion of LacZ$^+$ pISP plasmids recovered during the excision assay. All plasmids were digested with *Eco*RI and *Hin*dIII. Digestion of pISP (lane a) and pISP2 (lane b) results in a 600-bp fragment containing the *P* element (arrow). pISw$^+$ (lane c), a pUC8 plasmid containing only a 47-bp fragment of the *Drosophila* white gene, and all LacZ$^+$ pISP plasmids (lanes d–g) did not have the 600-bp fragment containing *P* sequences. DNA size standards are in kb.

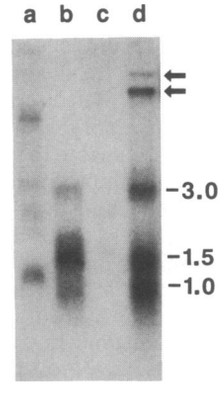

FIG. 3. Detection of *P*-element transcripts in *D. melanogaster* and *A. suspensa* embryos injected with pUChsπΔ2-3. Lanes: a, total RNA from 100 *Adh*^{fn23} *cn;ry*^{506} embryos; b, total RNA from 100 *Adh*^{506} *cn;ry*^{506} embryos injected with pUChsπΔ2-3; c, total RNA from 100 *A. suspensa* embryos; d, total RNA from 100 *A. suspensa* embryos injected with pUChsπΔ2-3. Transcript sizes are in kb. Arrows refer to plasmid DNA that is occasionally recovered during RNA preparation from injected embryos. The probe was a uniformly labeled 700-nucleotide single-stranded antisense RNA complementary to transposase open reading frame 0 and a portion of open reading frame 1.

tids but do not reveal the state of transposase message maturation.

DISCUSSION

We report data that indicate *D. melanogaster P*-element mobility *in vivo* to be phylogenetically restricted. Using a modification of the *P*-element excision assay of Rio *et al.* (14), we directly assessed *P*-element mobility in insect embryos. *P*-element excision was detected in all drosophilids tested, including species outside the genus *Drosophila*. With this assay *P*-element excision was found to be transposase dependent in all drosophilids tested except *D. melanica* and *Z. tuberculatus*, where spontaneous excision from pISP was observed with a frequency lower by a factor of 10 (5×10^{-5}) than when transposase was present. Because these species do not contain *P* elements (ref. 4 and unpublished observations) these excisions might be catalyzed by a functionally homologous protein encoded by another transposon system. Spontaneous excisions were not observed in *A. suspensa* and *T. curvicauda* or in the other drosophilids tested. Thus the *in vivo P*-element excision assay represents a sensitive method to monitor *P*-element mobility.

The frequency of *P*-element excision in drosophilids depended on the relatedness of the species to *D. melanogaster*—with distantly related species being less capable of supporting *P*-element mobility. Embryos of species outside the family Drosophilidae, *A. suspensa* and *T. curvicauda* (family: Tephritidae), were incapable of supporting *P*-element excision. Although drosophilids and tephritids diverged ≈120 million years ago (26) a number of other families are more closely related to Drosophilidae than are tephritids. Analyzing the mobility of *P* elements in representatives of these families should allow us to determine more precisely the limits of this phylogenetic restriction. Preliminary results of the excision assay conducted in species from families more and less related to Drosophilidae than are tephritids support our conclusion that *P* mobility is phylogenetically restricted. Significantly, these results suggest that without modification *P* elements will not be useful as gene vectors in Diptera other than drosophilids.

Although interest exists in the use of *P* elements as gene vectors in insects of economic and medical importance (27, 28), attempts to transform the germ line of the mosquito *A. gambiae* (10), and the Mediterranean fruit fly, *Ceratitis capitata* (family: Tephritidae; ref. 29), using *P*-element vectors carrying a dominant selectable marker (neomycin phosphotransferase) have failed to yield a *P*-element-mediated transformant. It is not known whether the failure of these experiments resulted from the absence of *P*-element sequences required for vector mobility in nondrosophilids, the lack of *P*-element insertion sites, the inability to confer whole-animal resistance to neomycin (in the case of *C. capitata*), or the absence of additional host-encoded biochemical factors required for transposition. Determining the mobility properties of *P* elements in heterologous systems has therefore remained an important unachieved goal.

Use of the *in vivo P*-element-excision assay to indicate *P*-element mobility in heterologous systems is subject to neither the limitations associated with using *P*-element vectors carrying a dominant selectable marker nor the reliance on germ-line transformation. (*i*) *P* elements carrying exogenous DNA are less mobile than unmodified elements, and this mobility is inversely proportional to the amount of DNA contained within the *P*-element vector (30). Such structural constraints might affect the mobility of *P* elements in heterologous systems to a greater degree than they do in *D. melanogaster*. For example, Brennan *et al.* (8), while trying to recover germ-line transformants of *D. hawaiiensis* using a *P*-element vector containing the *D. melanogaster* alcohol dehydrogenase gene, only recovered transformants containing autonomous, unmodified *P* elements. This result suggests that either structural features essential for *P*-element mobility in *D. hawaiiensis* were eliminated during vector construction or the presence of exogenous DNA more severely limits the mobility of *P*-element vectors in this system. (*ii*) The ability to confer whole-insect resistance to neomycin has only been demonstrated in *D. melanogaster* (9) and *A. gambiae* (10). The utility of this marker in other insects is not known, and its use in tephritids, which have high concentrations of symbiotic bacteria in their gut (31), is particularly problematic. Therefore, failure to attain germ-line transformation using a *P*-element vector carrying a dominant selectable marker has not allowed conclusions regarding the function of the *P*-element transposon system. The *in vivo* excision assay allows direct assessment of *P*-element mobilization without relying on genetic selection or germ-line transformation.

Most DNA injected into preblastoderm insect embryos is incorporated into somatic tissue (18), and as a consequence, *P*-element excision has been monitored in the soma. Nevertheless, our results show that functional transposase in the embryonic soma can support a level of *P*-element mobility comparable to that observed in germ-line tissue: the frequency of *P*-element excision from the pISP indicator plasmid (1.7 per 10^3 pISP plasmids recovered) is comparable to the reversion frequency (i.e., *P*-element excision) of the *P*-element insertion mutation $w^{hd80k17}$ (4 per 10^3 X chromosomes) occurring in the germ line of mutant *D. melanogaster* (1). The mutation $w^{hd80k17}$ consists of a 600-bp nonautonomous *P* element inserted into the coding region of the white gene of *D. melanogaster*. The nonautonomous *P* element and 47 bp of flanking white sequence from $w^{hd80k17}$ were used in constructing pISP (14). We believe the quantitative and qualitative similarities in *P*-element mobility in germ-line and somatic cells permit use of the somatic *in vivo* excision assay as a reliable indicator of *P*-element function in the germ line.

Recently Rio *et al.* (15), using the *P*-element excision assay, reported *P* mobility in mammalian cell lines producing transposase. These results are significant because they show that under certain conditions *P* elements can be mobilized in heterologous cells. However, *copia*-like elements, which are relatively immobile *in vivo*, have increased mobility *in vitro* (32), suggesting that in cultured cell lines control of transposable element mobilization may be generally altered relative to cells *in vivo*. Determining the basis of this mobility difference *in vivo* and *in vitro* should enhance our understanding of regulation of *P*-element function and our ability to fully exploit *P*-element vectors.

Understanding the basis of *P*-element dysfunction in nondrosophilids should reveal important aspects of the mechanism and regulation of *P*-element transposition and excision. Lack of *P*-element excision in tephritids is not attributable to failure of *P* transposase gene transcription. Helper plasmid pUChs$\pi\Delta$2-3 is transcribed in both *A. suspensa* and *D. melanogaster* embryos, and although some variability was seen in *P*-element transcript lengths in these embryos, transcripts of identical size were found in both species—with one transcript corresponding in length to a correctly processed transposase transcript. The transposase gene contains three introns, and although pUChs$\pi\Delta$2-3 lacks intron III, introns I and II are present and require splicing (16). Furthermore, intron II contains an alternative splice site, which, if used, will result in a four-amino acid deletion and nonfunctional transposase (16). Processing errors or use of alternative splice sites might account for the failure of *P* elements to be mobilized in tephritids. Thus, determining the extent of intron splicing of *P*-element transcripts in tephritid embryos or using helper plasmids that produce transcripts not requiring intron processing will inform us more precisely about *P*-element dysfunction in tephritids. Alternatively, the pres-

ence of negative regulatory factors or the absence of cellular components required as cofactors for *P*-element mobility may also result in immobilization of *P* elements. Species that do not support *P*-element excision will offer useful systems for testing putative host-encoded factors on *P*-element mobility.

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