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Invited review

A current perspective on insect gene transformation

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Abstract

The genetic transformation of non-drosophilid insects is now possible with several systems, with germ-line transformation reported in published and unpublished accounts for about 12 species using four different transposon vectors. For some of these species, transformation can now be considered routine. Other vector systems include viruses and bacterial symbionts that have demonstrated utility in species and applications requiring transient expression, and for some, the potential exists for genomic integration. Many of these findings are quite recent, presenting a dramatic turning point in our ability to study and manipulate agriculturally and medically important insects. This review discusses these findings from the perspective of all the contributions that has made this technology a reality, the research that has yet to be done for its safe and efficient use in a broader range of species, and an overview of the available methodology to effectively utilize these systems. Published by Elsevier Science Ltd.

Keywords: Insect gene transfer; Germ-line transformation; Transposable elements; Paratransgenesis; Viral vectors; Fluorescent proteins

Contents

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1. Introduction

Drosophila melanogaster was one of the first organisms to be routinely transformed, yet the extension of this technology to other insect species has been relatively slow. Despite considerable effort since *Drosophila* transformation was achieved in 1982, the first bona fide transposon-mediated transformation of a non-drosophilid germ-line was not reported until 13 years later. This was the *Minos*-mediated transformation of the Mediterranean fruit fly, *Ceratitis capitata* (Loukeris et al., 1995b). An accompanying commentary to this report ("Medfly Transformed-Official!") by Ashburner (1995) was indicative of the optimistic view that the longstanding barriers to genetic transformation of economically and medically important insects had finally been surmounted. This optimism is slowly but surely being validated, but just as the methodology for *Drosophila* transformation could not be extended simply to other insects, there was no reason to believe a priori that the transformation system first used in the medfly could be simply extended to more general use. Recent progress for other insects has actually resulted from a maturation of ideas and experimentation on a variety of facets of the transformation process that have come from a number of laboratories. This includes advancements in vector and marker systems, as well as new and modified techniques and equipment.

This review will focus on advancements that have made insect genetic transformation more widespread in recent years, and new and alternative methods to extend this technology to a variety of insect systems for specific applications. Most of the vectors used for germ-line transformation are derived from transposable elements, many of which have been discussed in a previous review in this series by O'Brochta and Atkinson (1996), and another informative review has recently considered the research needs and priorities for arthropod transformation (Ashburner et al., 1998). While very relevant information is provided in these reviews, rapid progress in achieving gene transfer, along with some unexpected results, make it timely to consider this subject once again.

Since several vector systems are already available for a range of insect species, we will also discuss some of the practical needs to achieve gene transfer. This should provide a starting point for the many laboratories who wish to take advantage of the new technology, and will allow a consideration of modifications that might be necessary for their insect system of interest. With progress in all phases of gene transfer technology, transgenic strains are already being planned and created for practical application, and risk assessment issues for their use in laboratory studies and potential release will be of utmost concern. This is a complex subject that will be reviewed briefly, though it should be one of the first subjects to be considered when planning a transformation experiment, and especially if release of transgenic insects is a possibility.

Some of the information discussed here was first reported at recent forums held to discuss advances in gene transfer technology in insects and other invertebrates. These include the Keystone Symposium, "Genetic Manipulation of Insects" held in Taos, NM, in January 1998, and the International Workshop on Transgenesis of Invertebrate Organisms most recently held at the Orthodox Academy of Crete, Kolymbari, Greece, in August 1999. Several of the speakers at these meetings have also contributed to a recent volume that reviews insect transgenesis, addressing many of the topics in greater detail (see Handler and James, 2000)

2. Background

The routine germ-line transformation of an insect species awaited the discovery of the *P*-element as the responsible agent for P–M hybrid dysgenesis in *D. melanogaster* (Kidwell et al., 1977), and the subsequent molecular analysis of its structure and function resulting

in the *P* vector and transposase helper system (Rubin et al., 1982; Rubin and Spradling, 1982). While there was considerable hope that *P* would have similar function in non-drosophilid insects, it was found to be non-functional outside of Drosophilidae (Handler et al., 1993). Nevertheless, the use of *P* in *Drosophila* has served as the theoretical and practical basis for most of the transformation systems currently in use for other insects — from vector and helper design to the way we inject embryos. There are clear exceptions and variations between the function of *P* and other transposon-based vectors now in use (referred to as the *P* paradigm; see O'Brochta and Atkinson, 1996), yet an understanding of the *P* element and its use for transformation in *Drosophila* is essential to fully appreciating other transposon and non-transposon gene transfer systems. It is thus worthwhile to briefly review the *P* vector as a model system, and several other reviews consider this subject as well in more detail (see Engels, 1989; Handler and O'Brochta, 1991).

P was found to be a 2.9 kb transposable element with short 31 bp inverted terminal repeat sequences, similar in structure to *Activator* (*Ac*), the first transposon discovered in maize by Barbara McCintock (see Federoff, 1989). In general, these elements (now known as Class II elements; see Finnegan, 1989) are approximately 1–3 kb in length with terminal sequences of 10–30 bp that exist in an inverted repeat orientation, though some elements have terminal repeats of several hundred base pairs. Within the termini a transcriptional unit encodes a protein known as transposase, which acts at or near the termini to catalyze a precise excision of the transposon and its subsequent re-insertion into another chromosomal site (known as a cut-and-paste reaction). *P* requires other nuclear factors as well to mediate transposition (Rio and Rubin, 1988), and the need, or lack of, for such factors and their host specificities probably plays a large role in the range of transposon function among insects.

Early experiments in *Drosophila* showed that a complete *P* element within a plasmid could transpose into the genome, with the subsequent finding that the transposase from this element could act in *trans* as a helper to catalyze the transposition of defective elements having intact terminal sequences, but a non-functional transposase gene. The introduction of an eye color marker gene, $rosy^+$, into a defective element created a vector whose transposition could be monitored in a mutant $rosy^-$ host strain, and this was the basis for the first binary vector/helper transformation system in *Drosophila* (Rubin and Spradling, 1982). *P*-mediated transformation, and all transposon-mediated systems developed since, now utilize a similar binary system where vector and helper plasmids are co-injected into preblastoderm embryos previous to pole cell formation. Plasmids introduced into the pole cell nuclei then have the potential to facilitate vector transposition into germ-line chromosomes. Some of the original transposase helpers were structurally complete autonomous *P* elements that could integrate along with the vector, thus increasing the chance for remobilization of the vector. This possibility was lessened by using greater vector to helper plasmid concentrations, but was only eliminated with new helpers having one or both termini deleted (referred to as "wings clipped") disabling the helper plasmid's ability to integrate (Karess and Rubin, 1984).

The early use of *P* for transformation of non-drosophilid insects has been reviewed previously (Walker, 1990; Handler and O'Brochta, 1991), but it is important to note that these attempts depended upon the availability of a drug-resistance selection system for transformants. This was the bacterial neomycin phosphotransferase II (NPT) gene placed into the *P* vector pUChsneo, that allowed transformed *Drosophila* to survive otherwise lethal doses of neomycin or its analogs such as Geneticin® (also known as G418) (Steller and Pirrotta, 1985). Extensive testing of pUChsneo in tephritid fruit flies, several mosquito species, and locusts failed to yield *P*-mediated transformants. While we now know that *P* is non-functional or highly restricted beyond drosophilid insects, assessment of the early transformations was additionally confused by NPT being an unreliable marker resulting in the selection of drug resistant insects that were not transformed.

The failure to achieve *P*-mediated transformation was frustrating, and especially so since the numerous variables in the gene transfer process made it difficult to do systematic control experiments to determine the limiting factors. Considering function of the *P* vector to be most critical, embryonic transient assays were developed to quickly assess *P* mobility in any insect embryo that could be injected. The development and results of these assays for *P* and other vector systems are discussed in detail by O'Brochta and Atkinson (1996), but suffice it to say that *P* activity decreased as a function of relatedness to *D. melanogaster*, with no mobility evident outside the Drosophilidae (O'Brochta and Handler, 1988; O'Brochta et al., 1991). The original assays that monitored transposon excision from a plasmid, and subsequent transposition assays that monitored transposon excision from a donor plasmid and insertion into a target plasmid, have become very powerful tools for the assessment of transposon vectors. All of the transposon vectors currently in use have been tested in one or more non-drosophilid species to verify their ability to be mobilized in a non-host species.

3. Transposon-mediated gene transfer

The most common form of germ-line transformation for non-drosophilid insects species utilizes transposon-

based vectors in a binary vector/helper system. While *P* and *hobo* vectors have been generally limited to drosophilids, four other transposon systems have transformed drosophilids and non-drosophilids. Table 1 lists all of the available transposon vector systems with published and unpublished references to the host species. Frequencies of transformation per fertile G_0 (injected G_0 embryos developing to fertile adults) are not given since accurate frequencies require repetition under consistent conditions, and most of these studies were unique and for some, grouped mating of G_0 insects precluded reliable determinations. Minimal frequencies, however, are in the range of 2–5% and no lower than 1%, with an upper range of 20–30% (usually for *D. melanogaster*). For most systems, variables affecting efficient transformation have not been optimized, with the most important including vector size, marker expression, and time of pole cell formation. Other critical factors such as DNA concentration, ambient conditions, injection needle preparation, among others are discussed further on.

3.1. Minos

The first transposon-mediated germ-line transformation of a non-drosophilid insect was achieved with the *Minos* element isolated from *D. hydei*. After its original discovery within the non-coding region of a ribosomal gene (Franz and Savakis, 1991) additional elements were isolated and sequence homology and general structure placed *Minos* within the *Tc* transposon family (Franz et al., 1994). *Minos* is a 1.4 kb element, that unlike the other Class II transposons discussed here, has relatively long inverted terminal repeats of 100 bp, and its transcriptional unit has a single intron. A functional *Minos* element was first used to transform *D. melanogaster* in several experiments, and *Minos*-mediated events were proven by sequencing insertion sites and remobilization of integrations (Loukeris et al., 1995a). The ability to test *Minos* transformation in a non-drosophilid was possible owing to the isolation of a cDNA clone for the Mediterranean fruit fly *white* gene (Zwiebel et al., 1995), which provided a selection for transformants in a *white eye* host

Table 1
Transposable element vector systems

Element	Host species	Species transformed	References
Hermes	*Musca domestica*	*D. melanogaster*	O'Brochta et al. (2000)
		Ae. aegypti	Jasinskiene et al. (1998) and Pinkerton et al. (2000)
		Tribolium castaneum	Berghammer et al. (1999)
		Ceratitis capitata	K. Michel and P. Atkinson, pers. comm.
		Culex quinquefasciatus	M. Allen and P. Atkinson, pers. comm.
		Stomoxys calcitrans	O'Brochta et al. (2000)
hobo	*D. melanogaster*	*D. melanogaster*	Blackman et al. (1989)
		D. virilis	Lozovskaya et al. (1996) and Handler and Gomez (1997)
		Bactrocera tryoni	S. Whyard, pers. comm.
Minos	*D. hydei*	*D. melanogaster*	Loukeris et al. (1995a)
		Ceratitis capitata	Loukeris et al. (1995b)
		Anopheles stephensii	Catteruccia et al. (1999b)
		D. virilis	L. Megna and T. Cline, pers. comm.
Mos1 (*mariner*)	*D. mauritiana*	*D. melanogaster*	Garza et al. (1991) and Lidholm et al. (1993)
		D. virilis	Lohe and Hartl (1996a)
		Ae. aegypti	Coates et al. (1998)
P	*D. melanogaster*	*D. melanogaster*	Rubin and Spradling (1982)
		D. simulans	Scavarda and Hartl (1984)
piggyBac	*Trichoplusia ni*	*Ceratitis capitata*	Handler et al. (1998)
		D. melanogaster	Handler and Harrell (1999)
		Tribolium castaneum	Berghammer et al. (1999)
		Anastrepha suspensa	Handler and Harrell (2001)
		Bactrocera dorsalis	Handler and McCombs (2000)
		Bombyx mori	Tamura et al. (2000)
		Pectinophora gossypiella	Peloquin et al. (2000)
		M. domestica	Hediger et al. (2001)
		Anopheles albimanus	O. Perera, R. Harrell and A. Handler, unpub.
		Ae. aegypti	N. Lobo and M. Fraser, pers. comm.

strain. Medfly transformants were created with *Minos* at an approximate frequency of 1–3% per fertile G_0 (Loukeris et al., 1995b). *Minos* transposition was subsequently demonstrated in dipteran and lepidopteran cell lines (Klinakis et al., 2000; Catteruccia et al., 2000a), with germ-line transformation reported for *Anopheles stephensii* (Catteruccia et al., 2000b) and *D. virilis* (L. Megna and T. Cline, personal communication).

3.2. *mariner*

The *mariner* element was first discovered in *D. mauritiana* in association with the somatically unstable *white*^{peach} allele (Jacobson et al., 1986; Haymer and Marsh, 1986), and was found to be a 1286 bp element with 28 bp inverted terminal repeats with four nucleotide mismatches. As with the *Tc* elements it has a TA target site specificity that is duplicated upon insertion. The original *mariner* was not autonomously functional, and its somatic activity was traced to a similar element called *Mos1*, having six different amino acids in the transposase transcriptional unit (Medhora et al., 1988). Similar elements were also discovered in other drosophilids, though interest in the element was heightened by the discovery of a distantly related *mariner* in the intron of the cecropin gene of the silkmoth, *Hyalophora cepcropia*. Surprisingly, thousands of copies existed in this species (Lidholm et al., 1991). Subsequent surveys for "*mariners*" throughout the Insecta, and more recently in organisms ranging from flatworms to humans, have been carried out by Robertson and his colleagues (Robertson 1993, 1997; Robertson and MacLeod, 1993; Robertson and Zumpano, 1997). Along with extensive functional analyses, *mariner* is one of the most completely studied transposon systems known to date and further details of this interesting story can be found in several comprehensive reviews (Hartl et al., 1997; Robertson and Lampe, 1995).

Of particular interest was the finding that autonomous *mariner* elements are widely functional and require no additional cofactors for mobility in vitro (Lampe et al. 1996, 1998). Yet, regulation of its activity is not straightforward and it is currently difficult to assess its general utility for insect transformation. Despite the high somatic activity of *Mos1* in *D. mauritiana*, its initial use as a transformation vector in *D. melanogaster* yielded quite low rates of transformation relative to *P* and *hobo* (Garza et al., 1991; Lidholm et al., 1993). It was subsequently used to transform *D. virilis*, but also at low frequencies and with resulting integrations that were not immediately apparent (Lohe and Hartl, 1996a). It was thus encouraging to find *mariner* mobile in several insects (Coates et al., 1997) and subsequently able to transform *Aedes aegypti* at somewhat higher frequencies of about 4% per G_0 using a transposase helper under *hsp*82 promoter regulation (Coates et al., 1998). Nevertheless, the relatively low transformation rates are surprising since *mariners* are so widely spread, and *Mos1* has been found to be active in microorganisms such as *Leishmania major* (Gueiros-Filho and Beverley, 1997) as well as vertebrates including zebrafish (Fadool et al., 1998) and chickens (Sherman et al., 1998). A related element from the horn fly, *Haematobia irritans*, known as *Himar1*, is similarly intriguing since it is active in bacteria (Rubin et al., 1999) and human cell lines (Zhang et al., 1998), but after extensive efforts it has thus far failed to transform *D. melanogaster*, and somatic activity has only been observed at low levels (Lampe et al., 1996). Yet, for both *Mos1* and *Himar*, transposase activity has been demonstrated in vitro in the absence of any additional cofactors except for Mg^{++} (Lampe et al., 1998), and so it is difficult to define the factors limiting the mobility of these transposons. A particularly interesting phenomenon originally observed in in vivo tests with *Mos1* was an inhibitory affect by increasing transposase concentrations, known as overproduction inhibition (Lohe and Hartl, 1996b). This is noteworthy since most transformation systems try to optimize vector transposition by having transposase helper under strong heat-shock promoter regulation. Apparently for some systems this can be counter-productive.

While there are several commonalities between *Mos* and *Himar*, discrete internal positions have been found to dramatically restrict mobility in *mariner* vectors (Lohe et al., 1997; Lohe and Hartl, 1996c), while *Himar* requires no more then 60 bp of each terminus for in vitro transposition (Lampe et al., 1998). Taken together, while we know more about the regulation of *mariner* elements than for any other transposon family, a greater understanding is necessary if any of these elements will be useful for routine insect transformation. Part of the difficulty may be due to regulatory differences that are host specific. Many of the *mariner* vector integrations in *Drosophila* were found to be highly stable, and refractory to remobilization by transposase. Yet, remobilization of *mariner* integrations in *Ae. aegypti* has been possible (C. Coates and A. James, personal communication), and vector immobility is counter-intuitive to the vast level of apparent horizontal transmission. Active investigations of *mariner* function and regulation are ongoing, and highly efficient bacterial-based assays for *mariner* function (Lampe et al., 1999) have a good probability of revealing mutations that will increase the efficiency of the system, either by their direct use, or by revealing necessary modifications.

3.3. *hobo, Activator, Tam3 (hAT) elements*

When it appeared that *P* would be of limited use in non-drosophilids, attention was directed to other transposon systems found in *Drosophila*, such as the *hobo* element that had also been developed into an effective

transformation vector (see Blackman and Gelbart, 1989; Blackman et al., 1989). Though closely related *hobo* elements were found in several *Drosophila* species (Daniels et al., 1990), it was unknown if *hobo* would be any more effective as a vector in non-drosophilids than *P*. Some basis for optimism came from phylogenetic comparisons of *hobo* to other transposons and it was discovered that regions of homology existed in plant transposons, in particular *Ac* and *Tam*3 (Calvi et al., 1991; Feldmar and Kunze, 1991). This suggested that *hobo* may have a wider range of function than *P*, and that related elements might exist in other insects that could be developed into new vectors. Several groups took a similar approach towards isolating *hobo-Ac*-related elements using regions of highest amino acid sequence homology to create primers for PCR. Several elements were isolated (see O'Brochta and Atkinson, 1996) but notable among these were *Hermes* from *Musca domestica* (Warren et al., 1994), *Homer* from *Bactrocera tryoni* (Pinkerton et al., 1999), and *hopper* from *B. dorsalis* (Handler and Gomez, 1997), and these are now considered to be members of the *hobo, Ac, Tam*3 (*hAT*) family of transposable elements. Of these, functional elements have only been discovered for *Hermes*, which has been developed into an effective vector. *Hermes* is a 2739 bp element having 17 bp inverted terminal repeats, and similar to *hobo*, it creates an 8 bp duplication of its insertion site. Full length elements have been found in *M. domestica* strains throughout the world, though it has not been discovered in any other species. *Hermes* was first used to transform the germ-line of *D. melanogaster* which occurred quite efficiently using a transposase helper under *hsp*70 promoter regulation (O'Brochta et al., 1995), and subsequent transposition assays indicated that it should be effective in a wide range of species (Sarkar et al., 1997a). *Hermes* has since been used to transform *Ae. aegypti* (Jasinskiene et al., 1998), *Tribolium castaneum* (Berghammer et al., 1999), *Stomoxys calcitrans* (O'Brochta et al., 2000), *Culex quinquefasciatus* (M. Allen and P. Atkinson, personal communication) and *Ceratitis capitata* (K. Michel and P. Atkinson, personal communication). *Hermes* function in at least two distinct insect orders suggests that it will be widely applicable.

Of particular interest were the *Hermes* transformations in *Ae. aegypti* where sequence analysis of two integrations showed that precise integration of the inverted terminal repeats did not occur and that donor plasmid from within and outside the vector had integrated in a rearranged fashion. Insertion junction sites occurred at both internal *Hermes* DNA and pUC19 donor plasmid DNA (Jasinskiene et al., 2000). These were clearly not precise cut-and-paste integrations as observed in *Hermes* transposition assays in this species (Sarkar et al., 1997b) and in previous *Drosophila* transformations (O'Brochta et al., 1995), but nevertheless the integrations were transposase-dependent.

Transposition assays with *hobo* suggested that *hobo* itself might have at least a low frequency vector function in non-drosophilids (O'Brochta et al., 1994), though beyond *D. melanogaster*, published reports are limited to low frequency *hobo* transformation of the distantly related drosophilid, *D. virilis* (Lozovskaya et al., 1996; Gomez and Handler, 1997). While some early attempts in non-drosophilids were unsuccessful or inconclusive, transformation in the Queensland fruit fly, *Bactrocera tryoni*, using a G418 selection yielded several putative transformants at a frequency of 9% (S. Whyard, personal communication). Molecular analysis indicated, however, that similar to the *Hermes* transformants in *Ae. aegypti*, plasmid DNA had integrated along with the vector in all the integrations. For at least one of these, *hobo* integrated precisely at one terminus, with the other junction occurring with plasmid DNA, indicating only a partial vector-mediated event.

The mechanism(s) for the imprecise *Hermes* and *hobo* integrations have not been determined, though the investigators involved have speculated upon types of recombination that may involve preexisting chromosomal elements that are the same or related to the vector. Normal transposition is thought to involve pairing of the inverted termini, and the imprecise integrations may have resulted from the transposase acting to resolve an interaction between the termini of endogenous and introduced transposons. Whether these interactions are specific to *hAT* elements and/or particular species remains an important question. The types of integrations observed are certainly reminiscent of the *P* vector integrations originally observed in *Anopheles gambiae* (Miller et al., 1987). In general such transformants should still be useful for laboratory studies, though it is important to note that while imprecise integrations may not affect marker genes allowing transformant selection, they might disrupt other genes of interest within the vector. Thus, until the behavior of specific vectors in specific species is clearly understood, a determination of transgene integrity will be warranted for most non-drosophilid transformations. A clear drawback for basic studies is that it is unlikely that imprecise integrations will be remobilized, and thus less useful for various manipulations such as transposon-tagging and enhancer-traps. For practical application, and especially transgenic insect release, the stability of such integrations will be an important consideration, though the *Aedes* transformants have remained stable for more than 2 years (A.A. James, personal communication). Indeed, it may be argued that if recombination events are not simply reversible, as are most precise transposon-mediated events, they actually may be highly stable and preferable for released strains.

Another important recent finding about *hAT* elements that will influence their use is the demonstration of

cross-mobilization between *hobo* and *Hermes*. The existence of *hAT* or *hobo*-related elements in several species was originally inferred by the mobilization of *hobo* in transient excision assays in the absence of its transposase (Atkinson et al., 1993; Handler and Gomez, 1996). For several of these species, complete or partial *hAT* elements were subsequently identified, most notably *Hermes* in *M. domestica* (Warren et al., 1994). Only recently, however, have direct tests been made to determine if cross-mobilization is possible. Sundararajan et al. (1999) performed plasmid and chromosome-based excision assays showing that *hobo* transposase could catalyze *Hermes* and *hobo* excision equally well, though *Hermes* was much less effective in catalyzing *hobo* mobilization. Although this confirmed an earlier notion of transposon interaction, the actual proof of cross-mobilization between distinct elements within a broad and dispersed transposon family should have a significant influence on how we use these elements as vectors. A standard for germ-line transformation has been stability of the vector insertion, and routine use of *P* and *hobo* vectors in *Drosophila* has been facilitated by the existence of host strains devoid of active elements (M and E strains, respectively). If mobilization is driven by related elements that may not be detected simply by hybridization, then it may be difficult to predict vector stability in specific hosts without extensive excision and transposition assays.

3.4. piggyBac

As with several transposons discovered in *Drosophila*, the *piggyBac* element (originally called IFP2) was identified by its association with a mutation, but in this instance the element was the causative agent of FP (few polyhedra) mutations in a baculovirus passed through the *Trichoplusia ni* TN-368 cell line (Fraser et al., 1983). It is a Class II transposon, 2.5 kb in length, having 13 bp inverted terminal repeat sequences and a 2.1 kb open reading frame, and is part of a subclass of elements originally found in lepidopterans that insert exclusively into TTAA target sites (Cary et al., 1989). Upon insertion, the target site is duplicated with excision occurring only in a precise fashion, restoring the insertion site. Beyond this functional similarity, the TTAA elements share no apparent structural identities.

The *piggyBac* element was first used to transform the medfly, *C. capitata*, using the medfly *white* marked vector and a self-regulated *piggyBac* transposase helper (Handler et al., 1998). Transformation frequencies were relatively low (~3–5% per fertile G_0), but notably, these experiments demonstrated *piggyBac* transcriptional and enzymatic function in an insect order different from the original host. Up to this time all other insect transformations utilized dipteran vectors to transform the same or a different dipteran, with most using a heat shock regulated helper. *D. melanogaster* was subsequently transformed with *piggyBac* at a similar frequency, though use of an *hsp70*-regulated helper increased the frequency to 26% (Handler and Harrell, 1999). We have since used *piggyBac* to transform the Caribbean fruit fly, *Anastrepha suspensa* (Handler and Harrell, 2001) and the Oriental fruit fly, *Bactrocera dorsalis* (Handler and McCombs, 2000).

The vector *piggyBac* has also been tested in several other insect species, and for some, transformation was anticipated based upon positive transposition assays (Thibault et al., 1999). Notably it has been used for the first transformations of Lepidoptera, including the silkmoth, *Bombyx mori* (Tamura et al., 2000) and the pink bollworm, *Pectinophora gossypiella* (Peloquin et al., 2000). Transformation of a coleopteran, *T. castaneum*, has been reported as well (Berghammer et al., 1999), though this study awaits molecular verification. Preliminary evidence exists for transformation of the mosquito species, *Ae. aegypti* (M.J. Fraser and N. Lobo, personal communication) and *Anopheles albimanus* (O.P. Perera, R.A. Harrell, and A.M. Handler, unpublished). Presently, *piggyBac* is a widely used vector for non-drosophilid transformation, and given its range of function it is likely to be active in many additional insect species.

An interesting, fortuitous discovery in the Oriental fruit fly transformation experiments was the detection of 10 or more *piggyBac* elements in host strain genomes, with sequencing of 1.5 kb PCR products showing close identity to *piggyBac* from *T. ni* (Handler and McCombs, 2000). Given the autonomous function of *piggyBac* among insect orders, the existence of the element in distantly related species is not totally surprising. The known movement of *piggyBac*, however, must have occurred by horizontal transmission and relatively recently. We have preliminary evidence for *piggyBac* in other species, but no evidence thus far for diverged related elements as evidenced for the *mariner*/*Tc* and *hAT* families. If *piggyBac* is widely distributed, and especially as a conserved element, this will have important implications for its use in basic research, and more so for practical application. If *piggyBac*, or a related cross-mobilizing element, exists in a host species, then transgene stability may be compromised, especially in strains being mass-reared. Thus far, integrations in *B. dorsalis* have remained stable for more than 10 generations based on the transgenic marker phenotype (under small-scale rearing). Nevertheless, these particular considerations are not an overwhelming drawback since endogenous elements can be detected by hybridization and PCR, and cross-mobilizing systems detected by mobility assays. A more daunting consideration is the potential for horizontal transmission of the transgene into non-target organisms. Though transgenes should be non-autonomous (or immobile in the absence of transposase) they may be vectored fortuitously by intermediate organisms such as

baculoviruses (where *piggyBac* was first discovered) into non-target organisms having the transposase.

4. Viral and symbiont vectors

Most efforts have focused on development of transposon-based vectors for insects since they have been successful for *Drosophila*, and they provide methods for genetic analysis. While these systems are preferable for stable germ-line transformation, it is realized that for particular applications in particular insects, other gene transfer systems may be necessary if not essential. Despite our optimism for widespread use of some transposon vectors, successful transformation has only been demonstrated for a relatively few species, most in the Diptera and Lepidoptera, and possibly some important species or orders of insects will not be amenable to transposition. Some species have long generation times, oviposit only a few eggs, are ovoviviparous, or have eggs that are impenetrable without compromising survival. Some applications may require gene transfer in the field, the rapid spread of genes through a population, or require only the persistence of somatically expressed genes. These situations and others will benefit from alternative modes of gene transfer and expression, including viral systems and gene expression from transformed symbionts.

4.1. Retroviral vectors

Retroviral vectors have long been used in mammalian systems to introduce and express genes of interest, and this has succeeded *in vitro* and in vivo (see Eglitis and Anderson, 1988; Anderson, 1998). Similar to transposon vector development, viral vectors are typically made replication-incompetent by deletion of requisite genes that may be replaced by genes of interest. Infectious particle production is possible in packaging cell lines that supply the needed viral proteins. Until recently the use of such systems was limited to vertebrate species owing to limited host range, but now a vertebrate retrovirus with an expanded host range has been created and a previously identified retrotransposon in *Drosophila* has now been defined as an active retrovirus.

4.1.1. Pantropic retroviral vectors

The Moloney murine leukemia virus (MoMLV) was engineered with the vesicular stomatitis virus envelope glycoprotein (VSV-G; known as pseudotyping) which binds to phospholipid moieties in host cell membranes (Burns et al., 1993; Yee et al., 1994). Thus it does not require specific protein receptor molecules for infection, resulting in a widely expanded host range beyond vertebrates, and importantly, it can be concentrated to high titers required for injection studies. As with other replication-incompetent viruses, it can be safely produced and utilized with minimal biological containment concerns. Burns and her colleagues have used this pseudotyped pantropic virus system to deliver genes into a wide range of organisms from amoeba (Que et al., 1999) to cows (Chan et al., 1998), including insect cell lines (Matsubara et al., 1996; Franco et al., 1998; Teysset et al., 1998) and embryos (Jordan et al., 1998; Franco et al., 1998).

There are, however, current limitations to the general use of the MoMLV pantropic virus as a vector system. First, the virus must be in contact with a cell membrane and undergo endocytosis so that the virus particle can uncoat in an endocytic vesicle. It cannot traverse intervening cells, and must be delivered in high enough quantities to reach appropriate cell membranes. Second, the vector lacks a nuclear localizing sequence and infection must occur in dividing cells for nuclear entry. While the pantropic virus is quite useful for integration into embryonic somatic tissue and cell lines, it does not currently present an advantage for vector delivery into the germ-line since it must be injected into preblastoderm embryos as is done with transposon vectors. A potential benefit for the system is that it may allow stable integration into species less amenable to transposon-mediated transformation, and it might be useful for secondary integrations since it would not interact with previously integrated transposon-based vectors. Efforts to overcome restrictions to use of the MMoLV pantropic virus include pseudotyping a lentivirus that contains a nuclear localizing sequence, precluding the need to infect dividing cells (see Burns, 2000). If infection of oogonia or spermatogonia in adults were possible, this could be a powerful method of vector introduction, possibly allowing transformation of many species not amenable to embryonic microinjection.

4.1.2. Insect retroviruses

Retroviruses specific to insects might circumvent the need to pseudotype, though until recently this viral group was not evident in insects. The *gypsy* element from *D. melanogaster* was first described as a retrotransposon (Bayev et al., 1984), though later it was found to encode an envelope protein, and was capable of infection. It has thus been reclassified as a retrovirus, the first to be described in insects (Kim et al., 1994). The infectious and transposition properties of *gypsy*, however, depend upon permissive alleles of the *flamenco* gene (that allow *gypsy* to "dance") (Prud'homme et al., 1995), and while *gypsy* proviral integration has been detected in the germline of permissive hosts, its rate of infectivity has been relatively low, limiting its potential for practical use.

Subsequently, other insect retroviruses/retroelements have been discovered, and while most have not yet been shown to be infectious, the *ZAM* and *Idefix* elements have been mobilized within the RevI strain of *D. mel-*

anogaster (Desset et al., 1999). Their copy number has increased within this line, with *Idefix* demonstrating a high level of target site specificity. Thus the potential exists for development of insect retroviral vectors from these elements or new ones, which should be explored in the near future.

4.2. Other viral systems

Several other insect viral systems have a potential for gene transfer in insects, and have already demonstrated the ability to achieve long-term transient expression after cellular infection (Carlson et al., 1995). Densoviruses are DNA viruses that are members of the parvovirus family discovered in mosquito and moth species, most notably in *Ae. aegypti* (AeDNV) (Buchatsky, 1989) and *Junonia coenia* (JcDNV) (Dumas et al., 1992). They have the potential to act as pathogenic biocontrol agents, or as vectors for gene transfer depending on structure, and while both have been shown to be infectious in other species, their host range is somewhat limited. Importantly, AeDNV vectors can be transduced into host organisms by typical routes of infection that greatly simplifies gene transfer, especially into populations if desired. Their primary limitation as vectors is that they are not likely to result in stable genomic integrations, though they may be used as carriers to introduce transposon-based vectors into organisms. This may prove useful to insects not amenable to DNA injection, or where maintaining the presence of weak transposon systems may allow transposition to occur. Densoviruses are also limited by the amount of DNA they can vector, currently in the range of four to six kilobases. Much remains to be learned about densoviruses, but they have significant potential as alternative vector systems or carriers.

Another viral system that efficiently transduces mosquito cells is the RNA Sindbis virus, (SIN) resulting in long-term stable, but thus far, only transient expression (see Olson et al., 1998). The virus has a relatively broad host range and can be introduced by feeding as well as by inoculation. Sindbis supports high levels of RNA transcription and protein synthesis in host cells that provides the potential for their acting as delivery systems for anti-pathogenic molecules in disease vectors. Similar to previously described viral systems, they are highly useful in basic studies of effector molecule function and may facilitate the use of weaker systems that allow stable integrations. At present Sindbis is most effectively used as a highly efficient transient expression system (see Higgs et al., 1996) and it has already found application in the analysis of the *Drosophila Ultrabithorax* gene in a butterfly and beetle (Lewis et al., 1999).

4.3. Paratransgenesis

For particular applications in particular species, germline transformation or typical transient expression systems may not be efficient or even possible. This might include species refractory to laboratory rearing or having unusually long generation times, and applications requiring the transgene or genetic modification being driven into a population. Several approaches are being taken to address these situations by using symbiotic organisms to express foreign genes of interest in a host insect, known as paratransgenesis. The most widely known mechanisms of paratransgenesis include two bacterial endosymbionts, *Wolbachia*, that is found in a variety of arthropod species (Sinkins et al., 1997), and *Rhodococcus rhodnii*, a symbiont of the kissing bug, *Rhodnius prolixus* (Beard et al., 1992). *Wolbachia* may be especially useful for driving genes through a population since it is known to spread rapidly by means of cytoplasmic incompatibility (Yen and Barr, 1973; Curtis and Sinkins, 1998). When uninfected females mate with infected males, sterility or partial sterility occurs, but when infected females mate with uninfected males or if both sexes are infected, fertility remains normal. Thus infected females have a reproductive advantage with an increase of infected organisms occurring each generation until, under optimal conditions, an entire population may be infected. Since this represents a positive drive mechanism, *Wolbachia* expressing a desired trait could be seeded in a small population of insects that upon release, could eventually spread throughout a field population (see Turelli and Hoffmann, 1999).

There are various caveats associated with this mechanism that vary with the species of interest, and incorporation of some types of transgenes may have negative affects on the symbiont or its transmission. One characteristic of *Wolbachia* having a negative or positive affect, depending upon the application, is the interspecific transfer of the symbiont between distantly related insects. The molecular mechanism for cytoplasmic incompatibility is unknown, and clearly this knowledge would greatly enhance the use and manipulation of the system. The major current impediment to the routine use of *Wolbachia* is the absence of methods to efficiently transform it with genes of interest.

Another type of paratransgenesis has been achieved in *Rhodnius prolixus* as a means to control the parasitic protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease. It was found that antiparasitic agents expressed by a bacterial endosymbiont of *R. prolixus*, *Rhodococcus rhodnii*, could decrease the ability of the insect host to transmit the parasite (Beard et al., 1998). A shuttle plasmid carrying the cecropin A antimicrobial peptide was created and used to transform the bacterial symbiont, that was subsequently used to infect host insects (Durvasula et al., 1997). This resulted in a significant reduction of trypanosomes within the host gut. New strategies include the development transgenic symbionts that express antibodies that specifically target the parasite (Durvasula et al., 1999), investigation of new

bacterial symbionts, and development of new bacterial vectors based on a mycobacteriophage that are more stable and efficient (E. Dotson and C.B. Beard, personal communication). It is notable that paratransgenic strategies are particularly suitable for species such as *R. prolixus*, which has a generation time of 6 months, making transposon and viral-based systems impractical to test at present.

5. Gene targeting

5.1. Homologous recombination

Gene targeting by homologous recombination has become an effective approach towards the transformation of several lower eukaryotes as well as plant and vertebrate systems (see Bollag et al., 1989). It has the major advantage of gene replacement allowing the efficient creation of null mutations or "knock-outs", or the restoration of normal allele function as well. Homologous recombination has been reported for *Drosophila* (Cherbas and Cherbas, 1997) and mosquito (Eggleston and Zhao, 2000) cell line studies, but only recently has it been demonstrated in vivo where it was mediated by a baculovirus in the silkmoth, *B. mori* (Yamao et al., 1999) and used in an experimental system in *Drosophila* (Rong and Golic, 2000). In the *Bombyx* study female moths were infected with a recombinant virus having the polyhedrin gene of the *Autographa californica* nucleopolyhedrovirus replaced with a silkmoth fibroin light chain–GFP gene fusion. Progeny of these moths had the gene fusion integrated into the resident light-chain gene, which was stably inherited and expressed in the silk gland. While not as efficient as transposon-mediated transformation, which has recently succeeded in the silkmoth as well (Tamura et al., 2000), this procedure clearly has the advantage of targeting genes for replacement, and especially for the creation of null mutations which will have many applications for insect genetics. Presently this procedure is most likely to be effective in insects subject to baculovirus infection, though conceivably the other viral systems discussed will have a similar potential as DNA carriers.

A recent attempt at homologous recombination in *Drosophila* was based on the premise that a linear extrachromosomal molecule could be recombinogenic with chromosomal DNA having homologous sequences (Rong and Golic, 2000). This was achieved by using the *FRT*/FLP site specific recombination system (see below) to create DNA circles by recombination between *FRT* direct repeats. The circles were linearized at a rare *I-SceI* 18-base endonuclease recognition site placed in between the *FRT* sequences. After inducing FLP recombinase and *I-SceI* endonuclease activity from genes integrated into the genome, the *Drosophila yellow* marker gene placed in the recombinogenic molecule was found to integrate into its chromosomal site with high efficiency in the female germ-line. This type of system has potential utility in any species that can be stably transformed, and would be of enormous benefit to the genetic analysis of insects.

5.2. Site-specific recombination

Previous to its recent use for homologous recombination, site-specific recombination systems have been used for various types of gene targeting and chromosomal manipulation. These systems include the *FRT*/FLP system from the 2 μm circle of yeast (Senecoff et al., 1985), as well as the bacteriophage Cre/*lox* system (Hoess et al., 1985). Both systems are functional in higher eukaryotes including *Drosophila*, with recombination occurring between specific sequences in the presence of a recombinase enzyme (Golic and Lindquist, 1989; Siegal and Hartl, 1996). For example, a functional *FRT* sequence site consists of two 13 bp inverted repeats separated by an 8 bp spacer, which efficiently and specifically recombines with other *FRT* sites in the presence of FLP recombinase. Depending upon their relative location, recombination can occur within and between chromosomes resulting in most types of chromosomal rearrangement (Golic and Golic, 1996; Golic et al., 1997).

A potential use for these systems in transgenic insects will be to delete or rearrange DNA within a transgene after chromosomal integration by specific positioning of recombination sites within a vector. Upon introduction or activation of recombinase, DNA necessary for mobility could be deleted or rearranged to immobilize the integrated vector for enhanced stability. In a similar fashion, markers or other DNA needed for gene transfer, but problematic for transgenic strain application, could be deleted from integrated vectors (Dale and Ow, 1991). The potential also exists for recombination systems being used for a new generation of vectors whereby an integrated recombination site is used as a chromosomal target by a plasmid vector carrying the same site and a marker. Recombination systems may be less vulnerable to cross-mobilization in eukaryotes, and specific targets can be selected that are less susceptible to position effect variegation/suppression. As primary systems for transformation become established, thereby allowing the integration of recombination sites into insect genomes and the creation of helper strains, site-specific recombination systems will provide many opportunities for genome manipulation.

6. Markers for selection of transgenic insects

6.1. Eye color markers and chemical selection

Insect transformation methodology has primarily focused on the discovery of functional vector systems, though as these have become available it is clear that the need for new marker systems is of equal, if not greater, importance. Advancements in *Drosophila* transformation have been due in large part to the availability of easily detectable eye color markers that are the wild-type genes for mutated alleles affecting eye pigmentation. The first non-drosophilid transformations also took advantage of available eye color markers, including the medfly *white* gene (Zwiebel et al., 1995), and the *Ae. aegypti* kynurenine hydroxylase-*white* mutation that is complemented by *D. melanogaster cinnabar*$^+$ (Cornel et al., 1997). Another eye color gene from *D. melanogaster*, *vermilion*, encodes tryptophan oxygensase and was used as a marker in early transformation experiments (Germeraad, 1976). It also complements the *green* eye color mutation in *Musca domestica* (White et al., 1996), and the tryptophan oxygensase gene from *Anopheles gambiae* complements *vermilion* (Besansky et al., 1997). While the discovery and elucidation of these various eye color genes is encouraging for their use as markers, in the absence of mutant host strains, such visible selection of transformed insects would be impossible for most species.

The need for dominant-acting selections independent of pre-existing mutant strains initially focused on genes that would confer chemical or drug resistance. These included NPT II conferring resistance to neomycin analogs (Steller and Pirrotta, 1985), organophosphorus dehydrogenase (*opd*) conferring resistance to paraoxan (Phillips et al., 1990; Benedict et al., 1995), and the gene for dieldrin resistance (*Rdl*) (Ffrench-Constant et al., 1991). Chemical selections can be very powerful, and if reliable, they would dramatically improve the efficiency of transformation screens for most insects by allowing selection en masse. However, most have been problematic due to protocols that vary with the treated species, and insects that are selected as untransformed false-positives. Since strain maintenance depends on resistance selection for each generation, the possibility of selecting for natural resistance mechanisms becomes greater with time. A possibility for utilizing the power of chemical resistance for mass screening would be the use of resistance markers in addition to a more reliable visible marker. Initial mass screens for G_1 transformants could be done by chemical resistance, with transformants verified and maintained as strains using the visible marker.

6.2. Fluorescent protein markers

Some of the most exciting dominant-acting marker systems to be developed recently are those using the green fluorescent protein (GFP) gene and its variants that include an enhanced GFP and spectral variants that emit blue, yellow, and cyan light (see the Clontech catalog for details). The GFP gene from the jellyfish *Aequorea victoria* (Prasher et al., 1992) first showed heterologous function in a nematode (Chalfie et al., 1994), and subsequently it was used as a reporter gene in *Drosophila* transformants as well as a variety of other organisms for both in vivo and in vitro studies. GFP was rarely used as a primary transformant selection, though this was probably due to available eye color markers and the need for UV optical systems for detection, which are not practical tools for mass screening. The more recent availability of fluorescent stereozoom microscopes now allows simplified screening of large numbers of insects.

To detect *piggyBac* transformants in the Caribbean fruit fly, which does not have a visible marker system, a GFP marker was developed having the enhanced GFP linked to a nuclear localizing sequence and regulated by the *Drosophila* polyubiquitin promoter (Handler and Harrell, 2001; see Davis et al., 1995). The marker was first tested in *D. melanogaster* using a *piggyBac* vector additionally marked with *white* as a positive control for transformation (Handler and Harrell, 1999). We actually found that GFP was more efficient than *white* since less than half the G_1 transformants detected by fluorescence exhibited visible eye pigmentation. Other GFP marker constructs have been developed including the use of the *Drosophila* actin 5C promoter to select *Hermes* and *Minos* transformants in mosquitoes (Pinkerton et al., 2000; Catteruccia et al., 2000b), and the *Bombyx* actin A3 promoter to select *piggyBac* transformants in *B. mori* (Tamura et al., 2000). GFP regulated by promoters that are active in all cells throughout development provide the added benefit of allowing selection of transformants as late embryos or larvae, which is a major advantage for insects with long generation times.

Another GFP marker construct that will have wide applicability uses an artificial promoter (3xP3) derived from the rhodopsin gene from *Drosophila* (Berghammer et al., 1999). It expresses most strongly from the brain, eyes, and ocelli in adults, but can also be observed from several structures in pupae and larvae. Notably, some insertions result in intense fluorescence that can be observed in pigmented eyes and it has already allowed selection of transformants in *T. castaneum* (Berghammer et al., 1999) and *M. domestica* (Hediger et al., 2001).

Markers in addition to GFP will be critical for the use of multiple transgenes, or where reporter genes distinct from the transgenic marker are needed. The 3xP3 promoter has been linked to the yellow and cyan fluorescent proteins that should expand the number of independent marking systems available (E. Wimmer, personal communication). In addition, a new red fluorescent protein (DsRed; Clontech) has recently become available that was isolated from an Indo-Pacific sea anemone-rela-

tive *Discosoma striata* (Matz et al., 1999). It has emission and excitation maxima that make it the most easily distinguishable fluorescent protein from GFP and the GFP variants. We have linked it to the polyubiquitin promoter, and in embryonic transient expression assays and *Drosophila* transformants it is easily detectable and distinguishable from GFP (A. Handler and R. Harrell, unpublished).

GFP and the other fluorescent proteins are likely to be useful as universal marking systems, and it will only take some experimentation to determine the best regulatory system for specific insect hosts, and specific applications. Drawbacks to the use of fluorescent proteins include quenching from hardened or melanized cuticle making detection difficult, and autofluorescence from gut material (often insect food) or necrotic tissue that may be mistaken for GFP expression. While GFP is useful for selection of transgenic insects in early development, the time needed for zygotic expression and subsequent internal cyclization and oxidation required for fluorescence will delay detection. Screening of G_1 transgenic insects should be delayed until late or post-embryonic stages, though in subsequent generations maternal GFP can be detected in early embryos (Davis et al., 1995).

7. Practical considerations for transformation

At this point there is sufficient evidence to believe that germ-line integrations or transient somatic expression can be achieved successfully in most insect species. Insect scientists can now seriously consider whether this technology will be useful to answer their biological questions, or to manipulate a species for practical application. However, before transformaton can be attempted it must be understood that there are practical considerations to the methodology that are critical. Most important are the physical methods necessary to introduce the vector system into host cells, since methods successfully used thus far may not be easily applicable to most insect species. Given the equipment costs and effort needed to set up a gene transfer system, a practical question is whether the goals of the research justify the investment needed. This has been less of a concern for researchers thinking about gene transfer in systems such as plants, animals, and *Drosophila*, since standardized techniques exist that are generally applicable. When considering insects, however, the variations in physiology, structure, and development among insects affect the parameters for the type of DNA delivery, timing of delivery, and rearing protocols required for specific species. All of the successful germ-line transformations to date have delivered vector by microinjection into preblastoderm embryos, basically following the *Drosophila* protocols, and obviously the host insects tested and their close relatives should be amenable to similar transformation methods. However, there are species in which the non-lethal (or non-sterilizing) injection of DNA into eggs will be much more difficult, and these will require experimentation or alternative approaches.

For those who wish to test microinjection, the procedures available for *Drosophila* have been described extensively (see Spradling, 1986; Karess, 1985; Ashburner, 1989a,b; Handler, 2000), and for non-drosophilids methods are described in the relevant reports describing their transformation. Essentially the goal for preliminary tests would be the injection of buffer with food coloring into preblastoderm embryos with minimal leakage, and their survival as fertile adults. To achieve this, some laboratories have very sophisticated and expensive microinjection set-ups, yet an efficient system for initial testing can be assembled with a relatively small investment, using equipment available in most departments. The essential equipment includes a stereozoom dissecting microscope, with transmitted or direct illumination (depending on whether eggs are translucent or not, respectively), with a mechanical stage and a micromanipulator. An inverted microscope, or even a compound microscope with a low power objective and mechanical stage, can also be used, and most micromanipulators used by neurophysiologists are adequate. The manipulator is required to align the needle at the embryo injection site, with the actual injection achieved by moving the embryo into the needle with the mechanical stage. Delivery of buffer into the embryo can utilize sophisticated air-pulse injection systems, but this can also be achieved with a syringe and fluid-filled tubing. It should be emphasized however that micro-needle preparation, with tips of 1–5 μm, is critical to successful injection (prepared with needle pullers used by neurophysiologists for microelectrodes), and for difficult embryos, beveled needles are very advantageous.

Alternatives to microinjection exist, and some have met with some success in *Drosophila*. Foremost is the use of biolistics to bombard eggs with micropellets encapsulated by DNA, which is a routine procedure for plant transformation (Klein et al., 1987). The predecessor to this technique, called ballistics, was used to introduce DNA into *Drosophila* embryos (Baldarelli and Lengyel, 1990). While a single *P* transformant line was created, the technique never gained wide applicability, though modifications were tested for mosquito eggs resulting in transient expression of reporter genes (Miahle and Miller, 1994). Biolistics has been used for transient expression tests in specific tissues, in particular the testing of fibroin gene promoters in the *B. mori* silk gland (Horard et al., 1994).

The other major method considered for DNA delivery is electroporation, which also allows efficient transient expression of DNA in *Drosophila* (Kamdar et al., 1992) and other insect embryos (Leopold et al., 1996). Again,

though transformation has not been reported, the potential for application does warrant continued consideration. Other imaginative approaches may include the use of endosymbiont or viral carriers or alternative mechanical delivery systems such as maternal injection into ovaries (Presnail and Hoy, 1992) or hemocoel. For some of these techniques, reevaluation is warranted in light of the development of more highly efficent vector and marker systems than those originally tested.

8. Risk assessment and regulation

The ability to create transgenic insects will undoubtedly have an enormous impact on a wide variety of basic biological and genetic studies, though the primary motivation for the development of this technology has been for the creation of transgenic insects that can affect insect population size and behavior. For most of these applications the release of transgenic insects is anticipated, and this presents unique and complicated challenges in terms of the design of transgenes, and risk assessment for release programs (Hoy 1992, 1995). This is especially so considering the controversy associated with transgenic plants (Abbott, 1996), though actual and perceived dangers may differ for non-edible organisms, or where the transgenic organisms and their offspring die after release without reproducing further.

Risk assessment studies must be tied, at a minimum, to regulatory procedures necessary to permit the transport or release of a transgenic insect. Permit applications for all arthropods have been evaluated by the Transgenic Arthropod Team (TAT) administered by the USDA Animal and Plant Health Inspection Service (USDA/APHIS), and it has already considered applications for the release or transport of several transgenic invertebrates. Information about the regulatory process, procedures for permitting transgenic invertebrates, and permits being evaluated and those issued can be accessed at the web site, and additional information is available in a recent review by Young et al. (2000).

Suffice it to say that some of the basic risk assessment issues are similar to those for permitting containment, transport or release of non-transgenic exotic or pathogenic arthropods, which include whether the organism presents a danger to plants, animals, or the environment. Other primary questions must deal with the stability of the transgene and whether its loss from, or movement within, the host genome will cause the transgenic organism to become harmful or act in unexpected ways. Associated questions should relate to potential horizontal movement of the transgene into non-target organisms and the mechanisms by which this may occur. Other issues deal with containment within the laboratory, during transport, and dispersal in the environment — will its movement be restricted or enhanced by geography or climate? More practical issues relate to deployment of transgenic strains and its viability and behavior in the environment.

The primary focus for the APHIS TAT has been regulation of genetically engineered plant pests, but APHIS also has statutory and general regulatory authority to restrict the importation and movement of organisms and vectors of animal diseases. However, current APHIS statutes and regulations do not specifically address the issue of oversight for genetically engineered vectors of animal diseases (see web site for details). As of this writing, APHIS will process permits for the transport of transgenic disease vectors between containment facilities, though the regulatory procedures and permit process for field trials and release remain under development. Given the fast pace of the field, it is important that these issues be resolved in the very near future, and the scientific and public discussion necessary for widespread consensus and implementation has already begun (see Aultman et al., 2000).

To assist in this process, interested university and government scientists are developing draft guidelines for the containment of transgenic arthropods of public health importance under the direction of the American Society of Tropical Medicine and Hygiene. These guidelines will provide advisory measures and principles of risk assessment for arthropods with respect to potential disease transmission, where the arthropod will be transported or contained, whether the arthropod is exotic, and issues specific to integrated transgene vectors and markers, among other considerations. Much of the content of these guidelines assigns various classes of arthropods to one of four containment levels that generally parallel, yet supplement, Biosafety Levels 1–4 that are described for pathogenic agents in the publication “Biosafety in Microbiological and Biomedical Laboratories” (US Dept. Health and Human Services, Public Health Service).

9. Summary

In the past several years genetic transformation of non-drosopohilid insects has made impressive strides resulting from advancements in all aspects of the methodology. Transposon vectors having no apparent host restrictions have succeeded in divergent species, and for some, in separate insect orders. Advancements for viral and symbiont vectors continue, and while germ-line transformation remains prospective for these, some are already finding laboratory and field application where somatic transient expression is sufficient, or even preferable. None of these vectors, however, could be tested without reliable marking systems, and indeed, the development of generally applicable markers such as GFP have probably had the greatest impact on recent

advancements in gene transfer. Clearly though, much remains to be done, with DNA delivery being one of the major current impediments to the more widespread application of insect transformation. All successful transformations to date have used microinjection into embryos, though for many insect systems this will be highly inefficient using current methods. Other DNA delivery techniques are available, including biolistics, electroporation, viral carriers, among others, though a concerted effort and possibly a group initiative will be required to carry out long-term systematic testing to make these reliable for important groups of species. Nevertheless, there is good reason to be optimistic that the technological hurdles for insect transformation will be overcome for most species.

The last major consideration for insect transformation is the application of this technology. *Drosophila* transgenics are now created in hundreds of laboratories, with the free exchange of strains worldwide that has resulted in incredible advancements in genetics research. While we look forward to similar advancements in our understanding of economically and medically important insects, the transport and release of most of these species will be subject to much more stringent regulation. These regulations will greatly impact the conditions under which we create and store transgenic insects, the choice and design of vectors and markers, and the testing and evaluation of transgenic insects for both laboratory use and field application. It is important at the early stage of this technology, to give the most careful consideration to the biological risk assessment and safety issues involved, and adherence to regulatory procedures as they are instituted. Only in this way will the greatest advancements and utilization of insect gene transformation be possible.

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