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## Transgenic mosquitoes: the state of the art

David A. O'Brochta<sup>#</sup>

### Abstract

After almost two decades of effort, the technology for creating transgenic mosquitoes has been developed. Although it requires specialized equipment, skills and facilities, any determined investigator can successfully use the technology. Delivery of transgenes to the germ cells of the host mosquito is accomplished by direct injection of pre-blastoderm embryos using fine, sharp glass needles. This delivery system is inefficient and demands special equipment and a high level of skill. Integration of the transgene into the chromosomes of the host insect is facilitated by the use of transposable-element-based gene vectors. *Hermes*, *mariner*, *Minos* and *piggyBac* are insect-derived transposable elements that have broad host ranges and function in mosquitoes. The efficiency of integration is between 1% and 10%; meaning 1-10% of the mosquitoes surviving the injection process and producing progeny will produce at least one transgenic offspring. Detecting transgenic mosquitoes is now very efficient with the advent of a wide variety of autofluorescent proteins that can serve as dominant visible genetic markers. The key question in the efforts to develop transgenic strategies for the modification of natural mosquito populations is not whether transgenic mosquitoes refractory to virus or parasite development can be created. Instead, the key questions now are: Can transgenic genotypes be successfully introduced into natural mosquito populations? Will the frequency of introduced transgenes in natural populations increase? Will the genetically modified populations of mosquitoes create the public-health benefit desired?

**Keywords:** Transposable elements, *Hermes*, *Mariner*, *Minos*, *PiggyBac*, mosquitoes, gene

### Introduction

Creating an organism containing foreign DNA that is stably integrated into chromosomes and transmitted to progeny provides opportunities to explore the biology of genetically manipulated organisms and to solve practical problems. These so-called transgenic organisms can pose risks to human health and the environment making their creation and use an issue of concern to people with a variety of interests. Given the central importance of mosquitoes to the health of a large fraction of the earth's population we can anticipate that the creation and use of transgenic mosquitoes will demand careful attention.

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<sup>#</sup> Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, Room 5115 Plant Sciences Building, College Park, MD 20742, USA. E-mail: obrochta@umbi.umd.edu

Transgenic insect technology was developing over the last two decades to the point where very determined investigators with a small amount of specialized equipment and the appropriate reagents had a reasonable hope of successfully creating a transgenic insect of their choosing. Interest in creating transgenic mosquitoes began two decades ago and followed reports of the development of the first insect-transformation system based on the *Drosophila* P transposable element in 1980. Unfortunately, that approach resulted in only limited success; see Handler (2000) for a more complete consideration of the history of the development of insect-transformation technologies. Miller et al. (1987) reported the successful creation of a single line of transgenic *Anopheles gambiae* that expressed an antibiotic-resistance gene. McGrane et al. (1988) and Morris, Eggelston and Crampton (1989) made similar reports of transgenic *Aedes triseriatus* and *Aedes aegypti*, respectively. The novelty of these insects at the time drew much attention, but the greater significance of the work was the demonstrated failure of the *Drosophila* transformation technology to function in a useful way in three mosquito species. These data and others (O'Brochta and Handler 1988) played a significant role in leading insect biologists away from the specific technology being used to create transgenic *Drosophila melanogaster* and to focus on developing alternative technologies. During the last decade there was a determined effort to develop alternative technologies that resulted in multiple functional systems for creating transgenic insects for a variety of applications. Herein I examine the current methods for creating transgenic mosquitoes and attempt to point out the limitations of existing technologies and what might be expected in the future.

### General Considerations

The term “transgenic” can be somewhat ambiguous. For the purposes of this discussion it will be taken to mean the heritable alteration of a genotype through the incorporation into chromosomes of DNA that was manipulated *in vitro*. This somewhat restrictive definition of “transgenic” is being employed because mosquitoes released into the environment as part of a disease-prevention programme in the future are likely to be transgenic in this way. This working definition of “transgenic” excludes organisms that may contain foreign genes extra-chromosomally, genes contained in viruses or microbial symbionts, as well as those organisms with foreign DNA in somatic tissue only.

Creating a transgenic organism requires the solution to three major problems. First, the DNA of interest must be delivered from a test tube in a laboratory to the nucleus of germ cells or presumptive germ cells of the target organism. Second, once in the nucleus the transgene must recombine with the chromosomes in such a way that it becomes physically incorporated into them. Third, germ cells containing the integrated transgene must be recognized and recovered. Solutions to the problems of transgene delivery, recombination and detection exist for a number of mosquito species permitting the creation of transgenic mosquitoes.

### Delivery

Creating transgenic mosquitoes requires the delivery of the transgene to the nucleus of germ cells or presumptive germ cells. Transgene delivery in insects has depended almost exclusively on the direct microinjection of DNA. Early insect embryogenesis in all mosquitoes and most insects, in general, involves a series of

nuclear divisions following male and female pronuclear fusion and then cellularization to form a blastoderm. Hence the early embryo is a syncytium and direct microinjection of DNA into the ooplasm is equivalent to direct cellular injection. As the nuclei in the young embryo proceed through the cell cycle the chromosomes are exposed to the injected DNA during mitosis and some of the DNA becomes passively incorporated into the reformed nuclei. Because it is known that the posterior pole of the embryo is where the presumptive germ cells will form it is possible to deposit the transgene-containing DNA in this region of the embryo to facilitate DNA incorporation into these cells. Following cellularization and blastoderm formation zygotic transcription begins and genes present on the microinjected DNA can be expressed.

Currently, direct microinjection of preblastoderm mosquito embryos using extremely fine glass needles is the only effective method for delivering DNA to germ cells for the purposes of creating transgenics. Alternative methods have been explored including biolistics (Baldarelli and Lengyel 1990; Mialhe and Miller 1994) and electroporation (Leopold, Hughes and DeVault 1996) of insect embryos but none of these methods proved particularly effective. Microinjection methods have improved through the development of appropriate protocols for collecting and handling embryos before and after microinjection. In addition, improvements in the manufacturing of glass microinjection needles, in particular the use of quartz glass, have permitted the resilient chorions of mosquitoes to be penetrated without destroying the needle or creating excessive mechanical damage to the embryo.

DNA delivery represents a significant limitation to the creation of transgenic mosquitoes. Newly-laid mosquito eggs have soft chorions that rapidly begin to melanize and harden, leaving researchers with a very small window of opportunity to perform microinjections. This window of opportunity can be extended in some species by treating the eggs with a phenoloxidase inhibitor, p-nitrophenyl p'-guanidinobenzoate (Catteruccia et al. 2000). In addition to a limited amount of time in which to perform the necessary microinjections young mosquito embryos are sensitive to desiccation. Embryos need to be slightly desiccated prior to injection to permit DNA being injected from leaking out. Mosquito embryos are also sensitive to small amounts of mechanical damage. Mosquitoes that deposit their eggs in rafts present additional challenges that can be overcome in some cases although this requires great skill and some innovation (Allen et al. 2001). Currently investigators with considerable skill at microinjecting insect embryos can expect to have approximately 10-30% of the mosquito embryos they inject survive and hatch.

## **Recombination**

The DNA delivered to mosquito germ cells is usually in the form of circular plasmids. Extrachromosomal DNA (that is, plasmids) will recombine with chromosomal DNA and this type of random recombination is the basis for creating stably transformed cell lines. The frequency of random recombination, however, is quite low and unless an efficient method exists for selecting for rare recombinants there is little chance of finding them. In addition, each mosquito progeny arising from a cross in which one of the parental insects had been injected with the transgene as an embryo reports on the genotype of one gamete from that parent. Screening tens of thousands of progeny probably represents a practical limit to what can reasonably be done in an average laboratory and, therefore, the frequency of transgene integration would need to be high enough to permit its recovery in a pool of tens of thousands of

gametes. Consequently, direct injection of plasmid DNA into mosquito embryos followed by random integration cannot be relied upon to create transgenics although such recombinants have been reported (McGrane et al. 1988; Miller et al. 1987; Morris, Eggelston and Crampton 1989). Instead, recombination needs to be promoted by attaching the transgenes to DNA sequences that are naturally highly recombinogenic. For those attempting to create transgenic mosquitoes, transgenes are attached to transposable elements.

Transposable elements are components of genomes that can change their location within a genome through the recombination reactions of excision and integration. Transposable elements are a structurally diverse group of genetic elements that can be divided broadly into two classes (Finnegan 1989). Class I elements transpose via RNA intermediates while Class II elements transpose via mechanisms involving only DNA and typically resemble cut-and-paste type recombination reactions. Class I transposable elements have not been developed into insect gene vectors. Class II transposable elements typically consist of a sequence of DNA less than 10 kb in length and often less than 3 kb with inverted repeat sequences defining the ends. These elements usually have limited protein-coding capacity, which usually includes a recombinase (transposase) that catalyzes the specific excision and integration of the element. Any DNA sequence flanked by the essential inverted terminal repeat sequences will behave like a transposable element in the presence of transposase; that is, they will excise and integrate. Hence the transposable element serves as a vector for the transgenes. The use of Class II transposons as gene vectors has become the standard strategy for promoting transgene integration into the chromosomes of insect hosts. Currently there are 4 transposable-element gene vectors that have been shown to function in mosquitoes: *Hermes*, *mariner*, *Minos*, and *piggyBac* (Handler and James 2000).

*Hermes* was isolated from the housefly, *Musca domestica* and is a member of the *hAT* (*hobo*, *Ac. Tam3*) family of transposable elements (Warren, Atkinson and O'Brochta 1994). It has a typical Class II transposable-element structure with a length of 2749 bp, 17bp inverted terminal repeats and a single open reading frame capable of encoding a 70kD transposase protein. Transgenic *Aedes aegypti* and *Culex quinquefasciatus* were created using this system and transformants were recovered at a rate of 1-5% (Coates et al. 1998; Jasinskiene et al. 1998).

*Mariner* (or *Mos 1*) was isolated from *Drosophila mauritiana* and is only 1.4 kb in length (Jacobson, Medhora and Hartl 1986). This element, which is a member of a widely distributed family of elements has been shown to be functional in a variety of taxa including protozoa, bacteria, fish, chickens, and insects. *Mariner* has been used to create transgenic *Aedes aegypti* with a reported rate of integration of approximately 4% (Coates et al. 1998).

*Minos* is a distant relative of *mariner* and belongs to the *TcI* family of transposable elements which has representatives in a wide range of taxa. *Minos* was originally isolated from *Drosophila hydei* and like *mariner* it is comparatively small, 1.8 kb, but possesses uniquely long terminal inverted repeats of 255bp (Franz and Savakis 1991). *Minos* was used as a gene vector in *Anopheles stephensi* (Catteruccia et al. 2000). The reported rate of integration in this species was approximately 10%.

*piggyBac* is a Class II transposable element that was isolated initially as an insertion sequence in a baculovirus that had been passaged through *Trichoplusia ni* cells (Fraser 2000). It was subsequently found that *piggyBac* originated in the genome of *T. ni*. *piggyBac* is the founding member of a unique family of elements referred to as TTAA elements and although their distribution has not been extensively

investigated members of this family are now known to be present in a wide range of insects including tephritid fruit flies (Hander, A.M. personal communication) and mosquitoes (Collins, F. personal communication). *piggyBac* has been used successfully to transform *Aedes aegypti* (Kokoza et al. 2000; 2001), *Anopheles gambiae* (Grossman et al. 2001), *Anopheles stephensi* (Ito et al. 2002; Nolan et al. 2002) and *Anopheles albimanus* (Handler, A.M. personal communication). In all cases the approximate rate of transformation ranged from 4 to 10%.

The variety of recombination systems that function in mosquitoes gives investigators a number of options for creating transgenic insects, and although the existence of these systems represents significant progress, technological limitations are apparent as well as gaps in knowledge. In combination with current DNA-delivery technologies the recombination rates associated with the transposable elements reviewed above are somewhat low. Rates of integration of 10% or less tend to be problematic and only are acceptable if producing viable injected parental insects is not difficult. Because DNA delivery to mosquito embryos is challenging, our ability to produce viable injected parental insects is limited. Consequently, increased rates of integration would make the technology more widely accessible. There has been little systematic analysis of the stability of the current gene vectors or their abilities to be intentionally re-mobilized, following their initial integration. For the elements *mariner*, *Hermes*, and *piggyBac* unexpected recombinants have been recovered that appear not to have arisen by the canonical “cut-and-past” transposition reactions that typify Class II transposable-element movement. The significance of these observations is not clear. Do unexpected integration events reflect properties of the elements or of the host?

## Detection

Most of the progeny will not be transgenic when they arise from a cross in which one of the parental insects had been injected as an embryo and was transformed. Insect germlines arise from tens of cells (pole cells) determined during early embryogenesis just prior to blastoderm formation and, therefore, any integration event occurring after pole-cell formation will lead to only a fraction of the gametes being transgenic. This percentage decreases as the timing of integration is delayed. It is not uncommon to find transgenic progeny comprising less than 1% of the offspring, and in some cases a single transgenic progeny is recovered suggesting that integration was very late in development and post-meiotic. Therefore, detection of transgenic individuals is a challenge. Fortunately, detecting transgenics is a general problem shared by all who create transgenic animals, plants and microbes. Consequently, there are a number of robust solutions to this problem. Two strategies currently employed for the detection of transgenic mosquitoes are mutation complementation and dominant visible phenotypes.

For well-studied species like *Aedes aegypti* and *Anopheles gambiae* there is a small collection of mutant lines with clearly visible phenotypes that can be used as parental strains in the creation of transgenics. By incorporating the appropriate wild-type allele in the gene vector one can recognize a transgenic individual by virtue of its now wild-type or near wild-type phenotype. The most useful mutant to date is an *Aedes aegypti* line with a recessive mutation in the kynurenin-hydrolase gene, a gene involved in eye-pigment biosynthesis. This particular mutation results in white (pigmentless) eyes and was originally described by Bhalla (1968). Introducing the wild-type allele of the *D. melanogaster* kynurenin-hydrolase gene results in partial rescue of the wild-type

phenotype. Rescued mosquitoes have eyes with varying intensities of red (Jasinskiene et al. 1998; Coates et al. 1998). Although mutant rescue is a useful method for detecting transgenic progeny, mutant lines can be less fit than wild-type mosquitoes and consequently may be more sensitive to the rigours of the injection process. In addition, the mutant genetic background may be inappropriate for subsequent investigations of the transgenic insect.

Introduction of a novel transgene that results in an easily recognizable phenotype has a number of advantages over mutant rescue. Often transgenic markers can be used with wild-type lines of mosquitoes. Currently there is a small collection of transgenic marker genes that result in a dominant visible phenotype. These marker genes were derived from marine invertebrates and encode for proteins that are autofluorescent. The proteins absorb light of a particular wavelength and then emit a photon at a wavelength that is readily visible. The most popular and widely used autofluorescent protein was isolated from the jellyfish, *Aequoria victoria*, and is fluorescent green (see Figure 1). A variety of modifications of the native green fluorescent protein (GFP) have been made that result in more intense light emissions and emissions at different wavelengths. Currently one can choose derivatives of GFP that emit yellow, green or blue light. Two additional autofluorescent proteins have become available that emit red light. Red fluorescent proteins were isolated from a sea anemone, *Discosoma striata* (DsRed), and a coral, *Heteractis crispa* (HcRed). Autofluorescent proteins currently available have proven to be valuable genetic markers for recognizing transgenic mosquitoes (Nolan et al. 2002; Pinkerton et al. 2000). The genes encoding autofluorescent proteins are small (about 1 kb) and can be placed under the regulatory control of a variety of promoters leading to widespread expression of the marker gene (Grossman et al. 2001; Nolan et al. 2002; Pinkerton et al. 2000) or under the regulatory control of tissue-specific promoters resulting in highly localized expression patterns (Ito et al. 2002; Kokoza et al. 2001). Although multiple robust systems for detecting transgenic mosquitoes are now available, none permit the easy distinction between a homozygote and a heterozygote. This technical limitation makes the establishment of true-breeding lines in the laboratory more difficult. Autofluorescent protein expression has been assumed to be essentially neutral to the mosquito although this assumption has not been tested and seems unlikely to be proven correct.

## Other Systems

Viruses have proven to be valuable for creating transgenic vertebrates but have not been used to date to create transgenic mosquitoes, as defined in this discussion. Sindbis viruses were used to transiently express genes in mosquitoes and are extremely useful tools for assessing gene function prior to investing the effort required to create a transgenic mosquito (Higgs et al. 1999; Olson et al. 2000). Sindbis viruses are expected to continue to play an important role in the molecular-genetic analysis of mosquitoes. Modified retroviruses were shown to have integrases that are functional in insect cells, but germ-line integration of these viral vectors has never been achieved (Matsubara et al. 1996). Densoviruses have also been used as transient expression vectors in mosquitoes and further development in this area is likely (Ward et al. 2001).

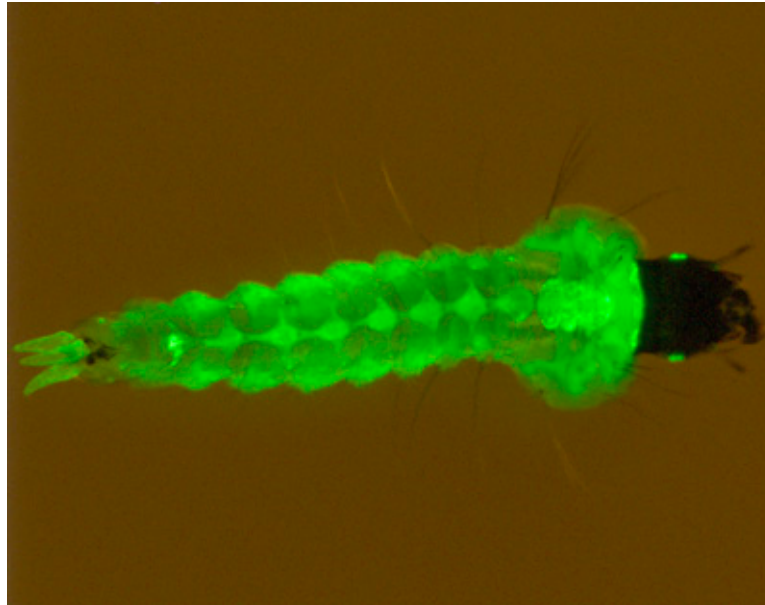


Figure 1. Transgenic *Anopheles stephensi* expressing EGFP.

## Conclusions

The creation of transgenic mosquitoes can now be considered an established methodology. The methods are not easy and require specialized equipment, skills and facilities but over time improvements can be expected that will result in this technology becoming more accessible. Improvements in DNA delivery and transposable-element integration rates are likely to be key points in the system that will be modified and lead to improvements. Despite the somewhat challenging nature of existing transgenic-mosquito technology it is being applied to questions of significant biological importance. The pace with which investigators create transgenic mosquitoes can be expected to accelerate rapidly in the near term. Already transgenic technologies are being used to explore potential strategies for disrupting parasite and pathogen development in mosquitoes. Antimicrobial peptides were introduced into *Aedes aegypti* (Kokoza et al. 2000) and a synthetic peptide was recently shown to have some disruptive effects on the development of *Plasmodium berghei* when expressed through the use of transgenic technology in the midgut of *Anopheles stephensi* (Ito et al. 2002). One can expect that antiviral and antiparasite strategies that were developed and tested through the use of virus-based transient expression systems will be placed into mosquito genomes using transgenic technology so that efficacy can be more realistically assessed (Adelman et al. 2001; De Lara Capurro et al. 2000; Higgs et al. 1998; Olson et al. 1996). We can expect that transgenic mosquitoes with a variety of genotypes and phenotypes will be created in the immediate future. Some of those transgenic mosquitoes will effectively and robustly block the development of pathogenic viruses and parasites. The key question in efforts to develop transgenic strategies for the modification of natural mosquito populations is not whether transgenic mosquitoes refractory to virus or parasite development can be created. Instead, the crucial questions now are: Can transgenic genotypes be successfully introduced into natural mosquito populations? Will the frequency of introduced transgenes in natural populations increase? Will genetically modified mosquito populations result in the desired public-health benefit? Developing the ecological

concepts that will aid in the development and assessment of new genetic control technologies is essential and needs to be ongoing.

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