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Gene Expression Studies in Mosquitoes

Xiao-Guang Chen^{*,1}, Geetika Mathur^{†,1}, and Anthony A. James^{†,‡}

^{*} Department of Parasitology, School of Public Health and Tropical Medicine, Southern Medical University, Guang Zhou, GD 510515, People's Republic of China

[†] Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697-3900, USA

[‡] Department of Microbiology and Molecular Genetics, University of California, Irvine, California 92697-4025

Abstract

Research on gene expression in mosquitoes is motivated by both basic and applied interests. Studies of genes involved in hematophagy, reproduction, olfaction, and immune responses reveal an exquisite confluence of biological adaptations that result in these highly-successful life forms. The requirement of female mosquitoes for a bloodmeal for propagation has been exploited by a wide diversity of viral, protozoan and metazoan pathogens as part of their life cycles. Identifying genes involved in host-seeking, blood feeding and digestion, reproduction, insecticide resistance and susceptibility/refractoriness to pathogen development is expected to provide the bases for the development of novel methods to control mosquito-borne diseases. Advances in mosquito transgenesis technologies, the availability of whole genome sequence information, mass sequencing and analyses of transcriptomes and RNAi techniques will assist development of these tools as well as deepen the understanding of the underlying genetic components for biological phenomena characteristic of these insect species.

I. INTRODUCTION

Mosquitoes are vectors of pathogens that cause serious human infectious diseases, such as malaria, dengue, and yellow fever. Increasing concerns with failures in existing control methods stimulate applied research, and genetic modifications of mosquitoes for population reduction and replacement are proposed as potential strategies to control mosquito-borne diseases (Curtis and Graves, 1988; Knols *et al.*, 2007). Basic molecular studies of mosquitoes reveal remarkable adaptations to facilitate hematophagy, reproduction, olfaction, and immune responses to pathogen challenge, as well as genetic bases for insecticide resistance (Attardo *et al.*, 2005; Enayati *et al.*, 2005; Meister *et al.*, 2004; Ranson and Hemingway, 2005; Ribeiro and Francischetti, 2003; Rützler and Zwiebel, 2005; Zwiebel and Takken, 2004). Both applied and basic science investigations benefit from advances in mosquito transgenesis technologies that include the discovery and development of transposable elements for germline integration of exogenous DNA, suitable marker genes such as fluorescent proteins, standardization of microinjection techniques, and characterization of promoters that drive tissue-, sex-, and stage-specific expression (Atkinson and James, 2002; Catteruccia *et al.*, 2000, 2005; Coates *et al.*, 1999; Conde *et al.*, 2000; Horn *et al.*, 2002; Jasinskiene *et al.*, 1998; Kim *et al.*, 2004; Kokoza *et al.*, 2001b; Lobo *et al.*, 2006). Furthermore, the availability of whole genome sequence information for three mosquito species, *Anopheles gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus*, allows global characterization of sequence conservation and genome

¹These authors contributed equally to the manuscript

structure through comparative and functional analyses by which patterns of evolution in gene and protein families are detected (Holt *et al.*, 2002; Nene *et al.*, 2007; Waterhouse *et al.*, 2008). This information is complemented by mass sequencing and analyses of transcriptomes, which provide information on gene expression levels in whole animals or specific tissues. RNAi techniques circumvent difficulties of conventional mutational analyses in mosquitoes and permit detailed studies of gene function and regulation. Targeted knockdown of transcription products in specific tissues is used in conjunction with bioinformatic gene discovery approaches to validate results *in vivo*. These advances hold great promise for the development of novel tools for controlling pathogen transmission as well as reveal the underlying genetic components for a number of biological phenomena found in these highly successful insect species.

II. STUDY OF GENE EXPRESSION IN MOSQUITOES

A. Transgenesis

Transgenesis, the stable integration of exogenous DNA into the genome of a target organism, is a powerful tool for gene expression studies and has been achieved with several mosquito species, all of which are important vectors of human pathogens (Table 2.1). Mosquito transgenesis is based on the paradigm developed for the fruit fly, *Drosophila melanogaster*, in which DNA flanked by the inverted terminal repeat (ITR) sequences of a Class II transposable element is mobilized (excised from a donor plasmid and inserted into target DNA, most often the mosquito chromosomal DNA). Mobilization is catalyzed by the action of the corresponding transposase encoded on a separate (helper) plasmid and usually under control of inducible, *cis*-acting promoter DNA. Mobilization assays monitor the movement of a transposable element from one plasmid to another and are important for determining if an element has the capability to excise and integrate in the embryonic environment of a specific species (Atkinson and James, 2002). These studies are a useful prelude before committing significant effort and resources to developing an element for transformation. Transgenes may contain DNA of homologous, heterologous, or synthetic origin, and their expression properties depend on *cis*-acting elements linked to marker, reporter or effector genes.

A number of early attempts at mosquito transgenesis supported dedicated efforts to develop systems that were both repeatable and reliable (McGrane *et al.*, 1988; Miller *et al.*, 1987; Morris *et al.*, 1989). The transposable elements *Hermes*, *Minos*, *Mos1*, *piggyBac*, and *Tn5* can integrate DNA into mosquito genomes and provide the bases for useful transformation vectors (Table 2.2). These elements have different evolutionary histories with *Hermes* belonging to the hAT family, *Minos* and *Mos1* derived from the mariner superfamily, and *piggyBac* representing a family in which it is the prototype. *Tn5* is of bacterial origin and belongs to the *IS50* family of insertion sequences. Wimmer and colleagues (Berghammer *et al.*, 1999; Horn and Wimmer, 2000) developed a set of donor transformation plasmids based on some of these elements that contain a variety of marker genes and the corresponding helper plasmids. Transformation efficiencies are best measured as the number of independent integration events that occur per fertile adult (Adelman *et al.*, 2002) and generally vary from 1% to 10%. However, higher frequencies are reported with *piggyBac*, especially in *An. Stephensi* (Adelman *et al.*, 2004; Kokoza *et al.*, 2001a; Nolan *et al.*, 2002). The elements integrate principally at their known consensus nucleotide sites (e.g., the dinucleotide, TA, for *mariner*-related elements), but no systematic studies of genomic integration sites are available, although it is reported anecdotally that they are random. *Hermes* has noncanonical integration behavior into chromosomes of *Ae. aegypti*, incorporating portions of the donor plasmid and deleting parts of the transgene, including the ITR sequences (Jasinskiene *et al.*, 2000). *Mos1* integrations into *Ae. aegypti* most often are single events while experiments using *piggyBac* in this species and

An. stephensi often recover animals with multiple insertions per genome (Coates *et al.*, 1998; Kokoza *et al.*, 2001a).

The apparent random nature of integration makes transgenes subject to insertion site effects that result in unanticipated expression characteristics. The expression profiles of multiple independent insertions must be analyzed to determine expression properties intrinsic to the transgene construct from those imposed by the surrounding genome. The use of “insulator” sequences to mitigate insertion site effects has been adopted widely in *D. melanogaster*, but has yet to be proven robust in mosquitoes (Farkas and Udvardy, 1992; Gray and Coates, 2004; Kellum and Schedl, 1991; Sarkar *et al.*, 2006). Site-specific integration into the genomes could mitigate these effects if a “docking site” were located in a region of the genome free of surrounding influences (Morris *et al.*, 1991). Chimeric *Mos1* and *piggyBac* transposases were reported to result in site-directed integration in plasmid-based transposition assays in *Ae. aegypti* embryos (Maragathavally *et al.*, 2006). However, the sequence complexity of target plasmids is much lower than genomic DNA, and it is not clear how the observed specificity extrapolates to mosquito chromosomes. The phage, $\Phi C31$, was exploited to develop a high-efficiency, site-specific integration system for inserting exogenous DNA into *Ae. aegypti* (Nimmo *et al.*, 2006). $\Phi C31$ uses a self-encoded integrase to insert its ~43 kilobase (kb) genome into a specific site, *attB*, of the host chromosome (Groth and Calos, 2004). Integration is mediated by synapsing *attB* with a specific sequence, *attP*, in the phage genome. The resulting recombination event produces two new sites, *attL* and *attR*, that are not substrates for the integrase, resulting in stable integration of the $\Phi C31$ genome. Linking *attP* or *attB* to any circular DNA fragment can facilitate its stable uptake into an *attB* or *attP* site, respectively (Thyagarajan *et al.*, 2001). Once docking sites were introduced into the *Ae. aegypti* genome, subsequent integrations were 7.9-fold higher than the primary integration event (Nimmo *et al.*, 2006). This approach should allow the insertion of large fragments of DNA into the mosquito genome.

Postintegration stability of nonautonomous (lacking a source of transposase) transgenes has not been a problem in general in mosquitoes. While one construct based on *piggyBac* was noticeably unstable in *Ae. aegypti* (Adelman *et al.*, 2004), it has proven difficult to induce remobilization of most transgenes once they are inserted into the genome (Sethuraman *et al.*, 2007; Wilson *et al.*, 2003). The reasons for this include disruptions of the element structure upon integration (Jasinskiene *et al.*, 2000), but for those that inserted intact it is not known and likely is specific to the element. Engineered elements based on *Mos1* and containing a source of transposase (autonomous) were able to catalyze the chromosomal insertion of other elements carrying *Mos1* ITRs, indicating the transcription and translation of functional transposase, but no movement of inserted elements were observed (Adelman *et al.*, 2007). While most nonautonomous transposable elements are stable in the absence of the homologous transposase, it is possible that endogenous sources of transposase may destabilize them. Methods have been developed for postintegration elimination of all transposon sequences in fruit flies (Dafaalla *et al.*, 2006; Handler *et al.*, 2004), but these have yet to be applied to mosquitoes. However, experiments with *Cre*-mediated recombination of integrated DNA show that specific sequences bound by *loxP* sites can be excised at high frequencies from the *Ae. aegypti* genome (Jasinskiene *et al.*, 2003).

The first successful use of a marker gene to detect repeatable mosquito transformation used a wild-type copy of the *D. melanogaster* cinnabar gene to complement a white-eye phenotype in a mutant strain of *Ae. aegypti* (Jasinskiene *et al.*, 1998). However, the most robust and widely-applied marker genes are those developed by Wimmer and colleagues comprising coding sequences for enhanced green fluorescent protein (EGFP), the cyan fluorescent protein (CFP), and *Discosoma* sp. red fluorescent protein (DsRed) controlled by the *D. melanogaster* *Pax6* eye-specific enhancer–promoter combination (Berghammer *et al.*, 1999; Horn and

Wimmer, 2000). Positive transgenic mosquitoes are screened easily by observing the specific fluorescence in the eyes or other nervous tissue with UV-microscopy (Fig. 2.1). The anal papillae often fluoresce, but the reason for this is not known. Fluorescent marker genes behave as complete dominant alleles making it difficult to distinguish visually animals that carry one copy from those carrying two or more. Autofluorescence can confound the use of some coding sequences as reporter genes. Green and red autofluorescence are detected in the accessory glands of both transgenic and wild-type male *An. stephensi* (Catteruccia *et al.*, 2005) and a weak green signal was seen in the thorax of the wild-type mosquitoes (Yoshida and Watanabe, 2006). Detection of transgene expression products using immunoblots or gene amplification procedures for the transcription products may be needed to verify engineered expression (Chen *et al.*, 2007).

B. Evaluation of mosquito gene expression

The majority of reported gene expression analyses in mosquitoes use technologies developed in the last century and include Southern blots to determine gene copy numbers, Northern blot analyses to determine the size and presence of specific transcription products, hybridizations *in situ* to examine RNA distribution and accumulation in specific tissues, gene amplification for detecting the presence/absence and abundance of transcription products, and primary sequencing analyses of both genomic DNA and cDNAs for intron identification and processing of primary transcripts. A growing number of more recent studies have exploited genome projects and mass sequencing to look at genome-wide transcription profiles. These studies reveal intricacies of the evolution of these highly adapted ectoparasites as well form the bases for developing novel control strategies. Functional analyses remain critical for establishing the roles of specific DNA sequences in gene expression and modulation.

1. Analyses of mosquito-derived control DNA—Mosquito transgenesis procedures, although generally routine, are not trivial. As a result, a number of transient methods were developed for assaying promoter functions in whole animals or dissected tissues. Transient expression assays were developed prior to the availability of transgenesis methods to look at gene expression in *Ae. aegypti* salivary glands (Morris *et al.*, 1995). A liposome-based transfection reagent was used to introduce a DNA construct comprising the luciferase reporter gene under control of the *D. melanogaster* HSP70 promoter into cultured adult salivary glands. Luciferase activity was detected in glands indicating that, although terminally differentiated, the glands could take up and support the expression of exogenous DNA. A more detailed analysis was done on the γ -aminobutyric acid receptor (GABAR)-encoding gene (*Rdl*) of *Ae. aegypti* that confers high levels of resistance to cyclodienes (Shotkoski *et al.*, 1996). *Cis*-acting DNA containing the *Rdl* promoter was mapped to a 2.53 kb fragment following transient expression of plasmid constructs in microinjected embryos. A detailed study was made of the *Ae. aegypti* ferritin light-chain homologue (LCH) gene in cultured cells (Pham and Chavez, 2005). Transfection experiments indicate that this gene has a strong promoter, and DNase I footprinting identified a number of transcription factor-binding sites, including those that bind GATA, E2F, NIT2, TATA, and DPE. More recently, a transfection assay based on DNA microinjection into whole animals was used to analyze a vitellogenin-luciferase reporter gene in response to blood feeding (Isoe *et al.*, 2007). A small, 843 base pair (bp), fragment of the *Ae. aegypti* vitellogenin-C promoter directed a >200-fold tissue-specific induction. The size of the control DNA is consistent with prior observations in transgenesis studies with an *An. stephensi* vitellogenin-encoding gene promoter (Nirmala *et al.*, 2006). Functional mapping of the *Ae. aegypti* gene identified essential 5'-end regulatory elements in the region -780 to -182 bp from the transcriptional start site. Similar assays in the same study revealed a 1096 bp genomic fragment of the cecropin B gene could induce > 100-fold expression in the fat body. These techniques need further development to be widely applicable for future gene analyses.

Mosquito genes of interest were defined first by their expression in tissues or body compartments in which significant interactions with pathogens were expected to take place (Meredith and James, 1990). Genes expressed in the midgut and salivary glands were isolated and characterized first. A number of efforts were made to assay mosquito *cis*-acting promoter DNA in *D. melanogaster* with variable success. A promoter fragment of the *An. gambiae* *D7r4* gene drove strong tissue-specific expression in the fruit fly, but only worked at a low level in *An. stephensi* (Lombardo *et al.*, 2005). Thus, transformation of the fruit fly is an unreliable assay for establishing expression properties of mosquito promoters. Following development of transgenesis, functional *cis*-acting control sequences were identified for genes encoding salivary gland secretory products, digestive enzymes, and proteins sequestered in the developing oocyte (Table 2.3).

Genes expressed in mosquito salivary glands encode proteins that facilitate feeding. While both sexes feed on sugars found in extrafloral nectaries, only adult female feed on blood and there are many genes whose expression is restricted to this sex. This latter category encodes a wide array of enzymes and other factors that counteract host hemostasis and immune system responses, and may initiate primary digestion of the blood (Ribeiro and Francischetti, 2003). For the most part, salivary gland gene expression appears constitutive, although some induction may be present after a blood meal to replenish stores (Marinotti *et al.*, 1990, 2005; Yoshida and Watanabe, 2006). Functional analyses of salivary gland gene promoters were stimulated because they were candidates for expressing antipathogen effector molecules. Genomic DNA fragments of the *Ae. aegypti* *Maltase*-like I (*Mall*) and *Apyrase* (*Apy*) genes were used to direct the expression of the luciferase reporter gene in transformed mosquitoes (Coates *et al.*, 1999). Both *Mall* and *Apy* promoter regions were capable of directing correct developmental-, sex-, and tissue-specific expression, but at low levels. In a separate study, an ~800 bp fragment of the *An. gambiae* *Apyrase* gene *AgApy* directed expression of a reporter gene in the salivary glands of transgenic *An. stephensi* (Lombardo *et al.*, 2005). Expression levels were low and the reporter gene product accumulated ectopically in the lobes of female salivary glands. Abundant expression in mosquito salivary glands was achieved only recently using a promoter from the anopheline antiplatelet protein (AAPP), a 30 K protein family gene in *An. Stephensi* (Yoshida and Watanabe, 2006). The amount of transgene product (DsRed protein) was calculated to be 25 ng per pair of salivary glands. This is ~5% of the total salivary gland protein level, and a > 1000-fold higher level of expression than reported for other salivary gland-specific gene promoter-reporter constructs (Coates *et al.*, 1999; Lombardo *et al.*, 2005). Unlike the endogenous AAPP mRNAs, whose abundance peaks at 48 h postblood meal (hPBM), accumulation of DsRed mRNA reached a high level as early as 24 hPBM and maintained this expression level for the subsequent 24 h. One explanation of this abundance of the DsRed mRNA may be due to a high stability of its mRNA.

Genes whose products are involved in digestion of the blood meal are induced within a short time after feeding. A number of trypsins, peptidases, other enzymes, and transport proteins are recognized as being involved in this developmental phase. There are early and late phases of digestion that vary among the different mosquito species in the specific timing and genes involved (Barillas-Mury *et al.*, 1995; Müller *et al.*, 1993). The best characterized genes are those that encode carboxypeptidases (CPs, Table 2.3). The endogenous genes are upregulated within a few hours after a blood meal and likely are involved in the primary digestion of the blood meal (Edwards *et al.*, 1997, 2000). Functional promoters have been characterized from genes derived from *Ae. aegypti* and *An. gambiae* (*AeCP* and *AgCP*, respectively; Kim *et al.*, 2004; Moreira *et al.*, 2000). While immunoblot data indicate that carboxypeptidase promoter-driven transgenes could result in the accumulation of ~2 ng of reporter gene protein per gut at 24 hPBM (Moreira *et al.*, 2000), the accumulation of heterologous gene products may be so low that it cannot be detected, and proper function of the promoter was established by gene amplification of mRNAs (Kim *et al.*, 2004).

A set of studies investigated the ability of CP genes to function in heterologous hosts. *Aedes aegypti* and *An. gambiae* bear only a distant evolutionary relationship and may have last shared a common ancestor 140–180 million years ago (Krzywinski *et al.*, 2006). Despite an apparent lack of sequence similarity between the *AeCP* and *AgCP* putative promoters, both can drive robust expression of a luciferase reporter mRNA and protein in a blood-inducible manner in *Ae. aegypti* (Moreira *et al.*, 2000). Interestingly, the native induction in *An. gambiae* of *AgCP* by a blood meal is rapid, 3 hPBM, but is much slower, 24 hPBM, when the sequences are integrated in *Ae. aegypti*. The *AeCP* promoter functioned in *An. gambiae* to drive reporter gene expression in the posterior midgut beginning ~24 hPBM (Kim *et al.*, 2004).

The fat body tissues of mosquitoes often express genes whose products are localized or transported through the hemolymph. The regulatory region of a vitellogenin-encoding gene (*Vgl*) of *Ae. aegypti* directed high levels of expression resulting in the abundant accumulation of the reporter gene product in the hemolymph of blood-fed female mosquitoes (Kokoza *et al.*, 2000). The reporter gene used in these studies encoded a defensin, and the protein persisted in the hemolymph of blood-fed female mosquitoes for 20–22 days after a single blood feeding. Control sequences of *AsVgl* directed blood meal stimulated sex- and tissue-specific expression of a reporter gene in transgenic adult female *An. stephensi* (Nirmala *et al.*, 2006). DNA fragments encompassing the 850 bp immediately adjacent to the 50-end of the gene and the 30-end untranslated region are sufficient to direct this expression. The control sequences of an *An. gambiae* vitellogenin-encoding gene, *VgT2*, directed correct expression of a reporter gene in a tissue-, stage-, and sex-specific manner in *An. stephensi* (Chen *et al.*, 2007). Furthermore, multiple blood meals resulted in persistent expression of the reporter gene, making the promoter a good candidate for directing the abundant accumulation of exogenous gene products.

Detailed analyses of transcription factor-mediated gene expression in mosquitoes have been carried out in *Ae. aegypti* by Raikhel and colleagues (Attardo *et al.*, 2003; Cho *et al.*, 2006; Park *et al.*, 2006). Following blood feeding, genes involved in the reproductive cycle, including those encoding yolk protein precursors such as vitellogenin and the vitellogenin receptor (*VgR*) are induced. *VgR* is involved in the receptor-mediated endocytosis of vitellogenin. A 1.5 kb fragment comprising 5'-end, putative *cis*-active *VgR* DNA was sufficient for correct female and ovary-specific expression of a transgene. The fragment includes binding sites for the products of the gene, E74 and BR-C, involved in the ecdysone response, as well as other sites for transcription factors determining correct tissue- and stage-specific expression. The 5'-end DNA of the vitellogenin-encoding gene has multiple sites for GATA-binding factors that are necessary for abundant expression. Remarkably, the products of a GATA factor-binding gene (*AaGATAr*) function as repressors prior to blood meal induction of vitellogenin-encoding genes. Of great interest was the demonstration that amino acid signaling through the nutrient-sensitive target of rapamycin (TOR) pathway is essential for the activation of YPP gene expression (Hansen *et al.*, 2004).

A number of other genes have been assayed functionally by transgenesis in mosquitoes. Much of the original work developing transformation vectors relied on promoters derived from *D. melanogaster*. Helper plasmids have the transposase open reading frames under control of heat shock promoters (HSP70 and HSP87) and a number of actin gene promoters have been used to drive the expression of marker genes (Catteruccia *et al.*, 2003; Pinkerton *et al.*, 2000). An EGFP marker gene under the control of the *act88F* gene promoter of *D. melanogaster* inserted into the genome of *C. quinquefasciatus* showed expression restricted to the flight muscle (Allen and Christensen, 2004). β 2-tubulin genes encode a protein that is expressed specifically in the male gonads. The promoter of the *An. gambiae* orthologous gene was used to drive EGFP expression in *An. stephensi* (Catteruccia *et al.*, 2005). Fluorescence was observed principally in the male gonads of the transgenic lines recapitulating the expression profile of the endogenous gene. Similar experiments were done with the β 2 orthologous gene of *Ae.*

aegypti (Smith *et al.*, 2007). As with the previous study, expression of a fluorescent reporter gene marked sperm that were detected in spermathecae of inseminated females. The *nanos* (nos) gene is expressed in females and is localized in the oocytes and is responsible for determining the anterior–posterior axis in developing embryos. The *nos* orthologous gene of *Ae. aegypti* was able to control sex- and tissue-specific expression of exogenously derived *MosI* transposase-encoding DNA (Adelman *et al.*, 2007). Transgenic mosquitoes expressed transposase mRNA in abundance and exclusively in the female germ cells. In addition, transgene mRNA was deposited in developing oocytes and localized and maintained at the posterior pole during early embryonic development.

Conditional and targeted expression of transgenesis in specific tissues can be achieved by using a tetracycline-regulated system. This system of conditional expression is based on transcriptional activators (TA) or reverse transactivator (rtTA) (Gossen and Bujard, 1992; Urlinger *et al.*, 2000) that respond to the antibiotic, tetracycline (tet), or an analog, doxycycline (dox). While TA binds to tet operator-derived response elements (TetO) in the absence of tet and permits transcription, rtTA binds in the presence of tet. This conditional expression system was tested in *An. stephensi* using *An. gambiae* SRPN10 promoter (Lycett *et al.*, 2004). Two transgenic lines were generated, one expressing TA or rtTA controlled by a tissue-specific SRPN10 promoter and another, expressing the *LacZ* gene under the control of TetO. Progeny obtained from a cross between the two transgenic lines show inducible *LacZ* expression in pericardial cells, hemocytes and epithelial midgut cells regulated by the SRPN10 promoter as well as by the presence or absence of dox. Surprisingly, a blood meal-specific increase in the percentage of *LacZ* expressing hemocytes was seen. Gene regulation by rtTA was stronger than TA in this study.

2. Expression of antipathogen effector genes—A major application of characterized *cis*-acting control DNA is to regulate the expression of transgenes that target specific mosquito-borne pathogens. The concept is that it should be possible to use transgenesis technologies to make mosquitoes resistant to a specific parasite or virus, and then use that mosquito in disease intervention strategies to control transmission (Meredith and James, 1990). Key to the successful development of these effector molecules is the utilization of *cis*-acting regulatory sequences that direct their expression to the pathogens at the right time and place, and in sufficient abundance to have an effect. The major pathogens that are targeted are the dengue viruses and malaria parasites. Dengue viruses have a positive-strand RNA genome, and a series of elegant studies have shown that these viruses are vulnerable to RNAi-mediated destruction (Sanchez-Vargas *et al.*, 2004). This work culminated in the production of transgenic *Ae. aegypti* that use the *AaCP* gene promoter to drive an RNAi-inducing transgene to reduce significantly the level of virus in mosquitoes (Franz *et al.*, 2006).

A wider variety of antiparasite effector molecules is available to attack malaria parasites (Nirmala and James, 2003). These include those that interfere with parasite ligands or mosquito-encoded molecules that facilitate tissue recognition by the parasite (pathogen “receptors”). Others interfere with parasite gene expression or elicit elevated insect immune responses. Finally, toxins also have been expressed that can differentially target the parasites. The most successful antiparasite effector genes used the CP-encoding promoters to target midgut-stage parasites. CP promoters activated <3 hPBM in the midgut can be used to interfere with parasite gametes, zygote and ookinetes. A 12 amino acid peptide, designated SM1, for salivary gland and midgut binding, was used to develop a transgene consisting of four SM1 units driven by the *AgCP* promoter (Ito *et al.*, 2002). SM1 expressed in transgenic females following an infectious blood meal bound to the luminal surface of the midgut, inhibiting significantly parasite-epithelium interactions and midgut invasion. Transgenic *An. stephensi* mosquitoes expressing a bee venom phospholipase A2 gene (PLA2) with *AgCP* as the promoter reduced parasite oocyst formation and greatly impaired transmission of the parasite to mice

(Moreira *et al.*, 2002). Follow-up studies with the regulatory DNA of the *An. gambiae* adult peritrophic matrix protein 1 (AgAper1) promoter fused to PLA2 led to the accumulation of PLA2 in midgut epithelial cells before a blood meal and its release into the lumen upon blood ingestion greatly affecting oocyst formation (Abraham *et al.*, 2005). Transient and stable transformation studies using single-chain antibodies (scFv) targeting important parasite ligands or other expression products have been shown to reduce intensities of infection of specific developmental stages (de Lara Capurro *et al.*, 2000; Yoshida *et al.*, 1999). Stable transgenics used mosquito vitellogenin-encoding (Vgl) and *D. melanogaster ubiquitin* gene promoters in parasite-infected *Ae. aegypti* (Jasinsktene *et al.*, 2007). These reagents have the benefit of targeting the parasite directly and thus are expected to not have a major negative effect on the fitness of the mosquito expressing them.

Manipulating the innate immune system of mosquitoes may be a possible approach to affect negatively their capacity to serve as parasite hosts. Immune system-based effector genes encoding antimicrobial peptides are effective against *Plasmodium in vitro* (Christophides *et al.*, 2002). Transgenes consisting of one of two representatives of the major peptide families comprising defensins and cecropins doubled the resistance exhibited by *Ae. aegypti* to the Gram-negative bacterium, *Enterobacter cloacae* (Shin *et al.*, 2003). Furthermore, transgenic *Ae. aegypti* strains overexpressing Defensin A inhibited oocyst growth of *Plasmodium gallinaceum*, and two independent lines of transgenic *An. gambiae* containing the *An. gambiae* cecA cDNA driven by the *AeCP* promoter showed a 60% reduction in the number of oocysts (Kim *et al.*, 2004; Shin *et al.*, 2003).

Maintaining large numbers of colonies, issues with pair matings and the need for blood meals at every generation make difficult standard genetic analyses with mosquitoes. Reverse genetic approaches, such as RNAi-mediated expression ablation, provide a way to circumvent these difficulties (Shin *et al.*, 2003). The assessment of gene function based on global expression patterns is often validated by a more direct method such as RNAi. Gene silencing can be achieved by injecting double-stranded RNA (dsRNA) into adult mosquito hemolymph (Blandin *et al.*, 2002) or by injecting recombinant Sindbis virus expressing specific dsRNAs (Attardo *et al.*, 2003). In the first study, dsRNA targeting transcripts of the antimicrobial peptide, defensin, showed the significance of this gene product in defense against infections with Gram-positive bacteria but not *P. berghei* infection, in contrast to the studies with *P. gallinaceum* infections of *Ae. aegypti* (Lowenberger *et al.*, 1999; Shin *et al.*, 2003). In the second experiment, knockdown of the *AaGATAr* gene revealed its role in regulating *Vg* gene expression and responses to 20-hydroxyecdysone.

Gene products can be knocked down efficiently in the mosquito fat body tissues and hemocytes. However, higher amounts of dsRNA are required to achieve knockdown in the salivary glands, presumably due to the poor permeability of the glands to nucleic acids (Boisson *et al.*, 2006). Injection of microgram quantities of dsRNA into *An. gambiae* showed that ablation of the products of *AgApy*, a gene encoding a platelet antiaggregating factor, apyrase, lengthened the duration of the probing behavior.

Genes that play important roles in vector-parasite interactions also are being identified using RNAi methods. The antiparasitic role of a complement-like protein, TEPI, was first demonstrated in *An. gambiae* by RNAi-mediated knockdown (Blandin *et al.*, 2004). Ablation of TEPI transcription products prevents a type of mosquito parasite refractoriness based on melanotic encapsulation of the pathogens. Another study showed that two mosquito genes had complementary effects, with WASP having a negative and ApoII/I a positive role in parasite development (Mendes *et al.*, 2008). Resistance to malaria parasite infection in a nonvector species *An. quadriannulatus* was shown to result from an innate immune response in which three genes, LRIM1, LRIM2, and TEPI, play a major role (Habtewold *et al.*, 2008). Knockdown

of these genes rendered the mosquito permissive to parasite infection. RNAi-mediated inhibition of the expression of the chitin synthase gene was used to probe the function of the peritrophic matrix (PM) in affecting pathogen invasion (Kato *et al.*, 2008). While PM loss did not have an impact on the development of the filarial worm, *Brugia pahangi*, or the dissemination of dengue virus, *P. gallinaceum* oocyst numbers were reduced. Interestingly, the absence of a PM had no effect on reproductive fitness. These studies emphasize the utility of RNAi approaches for dissecting complex mosquito phenotypes associated with development and vector competence.

Expression of transgenes in mosquitoes must not impose too great a genetic load if they are to be useful in control strategies. Two aspects of fitness could affect the success of a genetic control program based on transgenic mosquitoes, and for discussion purposes, they can be referred to as the “optimal” and “suboptimal” fitness of the transgenic strains (Scott, 2006). Optimal refers to those features of fitness that maximize the reproductive rate of the strain and are evident under laboratory rearing conditions in the absence of competition. Suboptimal conditions are present in the wild and include competition. All fitness estimates are relative and optimal reproductive success of the transgene-bearing lines must be referenced to the nontransgenic strain from which they were derived. In the simplest terms, female mosquitoes must be physically able to locate and feed on hosts, and recognize and use appropriate oviposition sites. Male mosquitoes must be able to locate and mate with females. Both sexes must be able to complete development without significant lethality in the adult and subadult stages. In addition to inbreeding depression, two other mechanisms associated directly with transgenesis, insertional mutagenesis and metabolic load caused by the expression of the transgene, may affect mosquito fitness. Insertional mutagenesis may cause a fitness reduction if integration of the transgene into the host genome results in the partial or complete disruption of an endogenous gene function at the insertion site. Expression of the transgene may be detrimental to mosquitoes if the gene product is toxic or gene translation usurps resources needed for normal reproductive functions. No studies have examined yet the effect of insertional mutagenesis and expression of the transgenes on mosquito fitness.

Fitness measurements of transgenic mosquitoes stimulate considerable debate (Marrelli *et al.*, 2006), and experiments performed to measure loads imposed by the insertion of exogenous genes yield data supporting contradictory conclusions. Catteruccia *et al.* (2003) found that *An. Stephensi* transformed with a *Minos*-based transposon construct with the *D. melanogaster Actin5C* or *hsp70* gene promoters driving the expression of EGFP exhibited a large fitness load in three of four transgenic lines in comparison to nontransgenic animals. Furthermore, *Ae. aegypti* transformed with a *Hermes* transposon carrying the *Actin5C* promoter linked to EGFP also exhibited significant fitness reductions (Irvin *et al.*, 2004). In contrast, Moreira *et al.* (2004) found that *An. Stephensi* transformed with a *piggyBac*-based transposon, EGFP reporter, and the AgCP promoter driving expression of the SM1 peptide effector gene had no detectable fitness load. The discrepancy in the results of these three studies was attributed to inbreeding depression (making homozygous one or more recessive alleles with negative fitness phenotypes) in that the Moreira study used hemizygous (one copy of the transgene, no alternative allele) transgenic animals to measure fitness, whereas the other two used homozygous individuals (Marrelli *et al.*, 2006). The nature of the expressed protein itself is a crucial factor for fitness. For example, although no effect on fitness was observed for mosquitoes expressing SM1, mosquitoes expressing PLA2 were clearly less fit and less fertile than wild-type (Abraham *et al.*, 2005). A recent report argues that there may be a tradeoff between fitness effects of the transgene and parasite infection (Marrelli *et al.*, 2007). Mosquitoes with transgenes expressing antiparasite molecules may outperform wild-type mosquitoes when both are infected with parasites.

C. Genomics

1. Comparative genomics—Comparative genomic analyses exploit whole genome sequences to identify novel and common genes among species, increase our knowledge of insect evolution, and complement the work done with single or small numbers of genes. In addition to gene discovery, analyses of common genes also can provide insights into mosquito gene regulation. Comparisons were done first with *An. gambiae* and *D. melanogaster*, which are estimated to have last shared a common ancestor some 250 million years ago (Holt *et al.*, 2002; Zdobnov *et al.*, 2002). While nearly half of the annotated genes appear to have orthologs in both species, sequence identity among these was ~56%. The authors point out that this number is lower than the number seen between humans and a fish that diverged ~450 million years ago. This was interpreted to indicate that these insects diverged at a higher rate than vertebrates.

The addition of the *Ae. aegypti* (Nene *et al.*, 2007) and *C. quinquefasciatus* (Vectorbase) genomes provides an opportunity to perform comparative genome analyses between mosquito species that diverged 140–180 million years ago (Krzywinski *et al.*, 2006). The genome size of *Ae. aegypti* is five times larger than *An. gambiae*, most likely as a result of a large number of encoded transposable elements and a higher content of repetitive DNA (Waterhouse *et al.*, 2008). Remarkable species-specific expansions of gene families encoding odorant-binding proteins, cytochrome P450s, secreted salivary gland proteins and genes whose products have domains characteristic of cuticle illustrate major differences among these organisms.

2. Functional genomics—Global gene expression analyses in mosquitoes are particularly useful in comparing expression profiles of nonblood-fed females before and after a blood meal, females and males, infected and noninfected mosquitoes and insecticide-resistant and susceptible strains. Genome-wide changes in expression levels were studied in the midgut of *Ae. aegypti* following a blood meal using cDNA microarrays comprising clones from expressed sequence tags (ESTs) (Sanders *et al.*, 2003). Not too surprisingly, significant regulation was seen in classes of genes involved in nutrient absorption and metabolism, cellular stress responses, ion balance, and formation of the peritrophic matrix. A larger-scale project with *An. gambiae* showed that as many as 33% of all annotated genes vary the abundance of their corresponding transcription products within 24h after a blood meal (Marinotti *et al.*, 2005). Major changes in transcript abundance were seen in genes encoding proteins involved in digestion, oogenesis, and locomotion. A comparison of gene expression profiles of *An. gambiae* adults at discrete times after a blood meal, in senescent adults and males was made (Marinotti *et al.*, 2006). Approximately 22% of the genes had sex-dependent regulation. Females devote the majority of their metabolism to blood digestion and egg formation within 3 hPBM and downregulate gene involved in flight and response to environment stimuli. The majority of changes in expression are evident over the first three days after a blood meal, when as many as 50% of all genes showed significant variation in transcript accumulation. After laying the eggs (between 72 and 96hPBM), mosquitoes return to a nongonotrophic stage, similar to that of 3-day-old nonblood-fed females. Data from these studies was compiled in a database for its use to study gene expression in a particular stage of the mosquito (Dissanayake *et al.*, 2006; Vectorbase). Additionally, algorithms to identify conserved *cis* regulatory motifs in genes coordinately expressed were included in the database. A proteomics approach using two-dimensional gel electrophoresis also was used to look at sex-specific and blood meal-induced proteins in *An. gambiae* midguts (Prévot *et al.*, 2003). Of ~375 observed proteins, samples from males showed ten not evident in sugar- or blood-fed females. Female midguts contained 23 proteins not found in males, eight of which were specific to sugar-fed and ten to blood-fed females. These data need to be followed-up with mass spectroscopy analyses to identify the specific proteins.

A detailed analysis of transcript abundance between the male and female *An. gambiae* midguts was carried out as well as for four regions in the female midgut (Warr *et al.*, 2007). Significant differences were found between the sexes in expression profiles and levels in genes involved in digestion and immunity. Furthermore, each anatomical region of the female midgut has a characteristic expression profile, for example, the posterior midgut expresses genes encoding digestive enzymes, while the anterior midgut expresses in abundance antimicrobial peptides and other immune gene products. The functional diversity of the female midgut observed was attributed to the requirements for dealing with blood meals and possible microbial infections that this feeding behavior would promote. Regional specification of the larval midgut also was demonstrated by microarray analyses (Ovieda *et al.*, 2008). The data support the conclusions that protein and carbohydrate metabolism and absorption take place in the gastric caeca and posterior midgut, while lipids are processed in the anterior midgut. Similar to the adult female midgut, transcripts corresponding to antimicrobial peptides and enzymes involved in detoxifying xenobiotics were localized to the gastric caeca and anterior midgut.

Bioinformatic and data mining searches were applied to the *An. gambiae* genome as soon as it was available. One of the more productive analysis identified genes encoding odorant-binding proteins (OBPs) and odorant receptors (ORs) (Biessmann *et al.*, 2002; Fox *et al.*, 2001). It is anticipated that this work could lead to the development of novel ways of preventing mosquitoes from finding and feeding on humans. Arrestins and *Gα*-encoding genes potentially involved in olfactory signal transduction also were identified. One of these ORs, AgOr7, is highly conserved in insects, and specifically in *An. gambiae*, and *Ae. aegypti* (Melo *et al.*, 2004; Pitts *et al.*, 2004). Sequence conservation and similar expression characteristics indicate an important and common olfactory function of these mosquito receptors. Expression in the proboscis of the orthologous genes in both mosquito species differs from the expression profile of the *D. melanogaster* gene, DOr83b, likely indicating adaptations to sensing different food substrates. Twenty-four OR-like gene products are located on the proboscis, which is consistent with a role as an accessory olfactory organ (Kwon *et al.*, 2006). The *An. gambiae* *Arrestin1* (*AgArr1*) rescued an olfactory deficit due to mutations of orthologous gene, *DmArr1*, in *D. melanogaster*, but the mutation-linked larval behavioral deficit was not rescued (Walker *et al.*, 2008). Additionally both *AgArr2* and *DmArr2* were unable to rescue the *DmArr1* mutation in fruit flies, suggesting a nonredundant function of the two different arrestins. In a different study, *An. gambiae* larval olfactory behaviors were examined using whole animal activity assays (Xia *et al.*, 2008). Strong responses in larvae were associated with compounds related to cresol that are produced from the decay of organic matter, which constitute the food of the larvae. These responses could be attenuated by ablation of the larval antennae. Four ORs were found that are specific to the larvae. Functional analysis of nine larval AgORs was performed with 82 odorants using *Xenopus* oocyte responses (Wetzel *et al.*, 2001). Multiple ORs responding to a single odorant indicate a combinatorial coding mechanism to encode odorant information in the larvae. Interestingly, both behavioral and oocyte responses of larval-specific AgOR40 to adult insect repellent, DEET indicate either the presence of additional DEET-sensitive receptors involved in adults or an indirect role of DEET as a behavioral repellent. Recently, a study revealed that insect ORs comprise a new class of ligand-activated nonselective cation channels (Sato *et al.*, 2006). ORs from *D. melanogaster*, *Bombyx mori*, and *An. gambiae* were expressed with their respective coreceptors in heterologous system where upon activation, they show increase in intracellular Ca²⁺. This activation does not involve G-protein signaling, providing evidence for a unique olfactory mechanism acquired by insects. Microarray-based studies of OBPs identified a number that are expressed at higher levels in either female or male antennae and palps (Biessmann *et al.*, 2005). Changes in OBP expression following a blood meal may reflect changes in the behavioral activity of females as the progress from blood feeding to nectar feeding.

Functional genomics also have been applied to define mosquito genes involved in interactions with malaria parasites. DNA microarrays were used to evaluate midgut responses to *P. berghei* and *P. falciparum* infections in *An. gambiae* (Vlachou *et al.*, 2005). Remarkably, >7% of the assessed mosquito transcriptome is regulated differentially following infection. Ookinete penetration of the midgut epithelium stimulates genes involved in actin- and microtubule/cytoskeleton-mediated and extracellular-matrix remodeling. Not surprisingly, other induced genes encode products involved in innate immunity and apoptosis. Coupled analyses with RNAi-mediated gene silencing identified antagonists and agonists of actin formation and provide evidence to support the conclusion that actin polymerization inhibits parasite invasion. Furthermore, the immune responses of the mosquito to the two parasites are quite diverse, and while some antimicrobial factors are induced in common upon infection, parasite-specific factors also are seen.

Infection by arthropod-borne viruses also stimulates changes in gene expression in mosquitoes. Although culicine mosquitoes (*Aedes*, *Culex*) transmit a large number of different flaviviruses (dengue, yellow fever) and bunyaviruses (Chikungunya, Rift Valley Fever), anopheline mosquitoes are rarely associated with virus transmission. A notable exception is o'nyong-nyong, and *An. gambiae* infected with this virus were assayed with microarrays carrying ~ 20,000 cDNAs (Sim *et al.*, 2005). Comparisons with uninfected mosquitoes revealed 18 genes whose expression levels varied significantly up or down. Although additional studies have to be done to identify the precise role of these genes in response to infection, the authors speculate that some of them may reflect efforts of the mosquitoes to resist infection or the action of the viruses to harness the biosynthetic machinery of the cells. Microarray analysis of *Ae. aegypti* infected with dengue viruses provides evidence that mosquitoes do respond to viral infection by inducing expression of genes involved in immune response, specifically those associated with the *Toll* pathway (Xi *et al.*, 2008). These authors also noted that the natural intestinal fauna of the mosquito may be a factor in attenuating dengue viral infection by providing a constitutive level of expression of genes in the *Toll* immune pathway.

Christensen and colleagues looked at expression patterns in mosquitoes infected with filarial worms (Aliota *et al.*, 2007; Bartholomay *et al.*, 2004). Many EST sequences from immune response-activated hemocyte libraries of *Ae. aegypti* and *Armigeres subalbatus* corresponded to immunity-related genes, some of which had strong similarity to genes involved in vertebrate innate immunity. In a follow-up study, the transcriptional response of *Ar. subalbatus* to *B. malayi* was shown to be complex and tissue specific.

Insecticide resistance in mosquitoes is a major factor driving efforts to develop genetics-based control strategies. However, insecticides still are the best tools available today to fight epidemic mosquito-transmitted disease outbreaks, and therefore there are compelling reasons to understand the molecular bases of resistance. This knowledge aids in particular the development of diagnostic tools to monitor resistance as part of programs that seek to manage resistance and therefore prolong the efficacy of specific chemicals. A cDNA microarray analysis of insecticide-resistant and susceptible *An. gambiae* detected 77 differentially transcribed ESTs (Vontas *et al.*, 2005). These included representatives of genes in expected families, for example, cytochrome P450s, a carboxylesterase, UDP-glucuronosyl transferases and nitrilases, and a number, including those encoding peptidases, ion exchangers and enzymes involved in lipid and carbohydrate metabolism, that were not anticipated to be linked to insecticide resistance. Furthermore, the array developed for *An. gambiae* could be used with reduced hybridization stringency to analyze resistance in *An. stephensi* (Vontas *et al.*, 2007). Although background was high, ~7000 significant signals were detected, 36 genes differed in expression levels among resistant and susceptible strains, including glutathione S-transferases, esterases, cytochrome P450s, and peroxidases.

One of the advantages of working with *D. melanogaster* is the wealth of information available on gene expression profiles throughout the development of the animal (<http://flybase.bio.indiana.edu/>). This type of information is now being put together for some mosquitoes, although much work still needs to be done. Spatial and temporal gene expression analysis of the *An. gambiae* life cycle reported genes involved with specific developmental stages (Koutsos *et al.*, 2007). Not too surprisingly, expression of genes known or annotated to be related functionally correlates with specific developmental stages or tissues. Furthermore, although a positive correlation was found for expression profiles among orthologous genes in the mosquito and fruit fly, the profiles did not correlate with coding sequence similarity. These data support the hypothesis that selection pressures allow independent evolution of expression properties and coding sequences.

Few detailed analyses have been made of individual mosquito genes involved in early development. While the expression profiles of the *nanos* and *oskar* (*osk*) genes are conserved generally, there are some differences noted in temporal and spatial distribution (Adelman *et al.*, 2007; Goltsev *et al.*, 2004; Juhn and James, 2006). Specifically, while mosquito *oskar* mRNAs were found to localize to the posterior end in early-stage embryos, *An. gambiae* also has transient localization in the anterior.

The development of rapid and inexpensive sequencing techniques now allows comprehensive analyses of transcriptomes of whole animals, development stages and isolated tissues. Millions of short cDNAs can be sequenced garnering information on transcript complexity and abundance. Perhaps no better example exists of the power of this approach than the efforts of Ribeiro and colleagues to define the “sialomes,” complete expression profiles of the salivary glands in mosquitoes and other hematophagous insects. Translation of most of the transcripts identified was confirmed by SDS-PAGE and Edman degradation. Female mosquitoes secrete a variety of proteins such as vasodilators, anticoagulants, and platelet anti-aggregating factors while taking a blood meal. Full knowledge of the transcriptome enables discovery of genes involved in various physiological processes associated with blood feeding. The data also provides insight into the evolution of the salivary gland protein families. The new genes discovered are too numerous to recount here, so the readers are referred to a number of key papers and a recent review (Calvo *et al.*, 2007; Francischetti *et al.*, 2002; Ribeiro, 2003; Ribeiro *et al.*, 2004, 2007; Valenzuela *et al.*, 2002). Key conclusions are that genes that encode proteins in phylogenetically widespread families are adapted for major roles in salivary gland function. Furthermore, there are salivary gland products derived from protein families present only in blood-feeding Nematocera. Within this group, there are families of proteins found only in the culicines or anophelines. Particular note is made of finding evidence of protein families of prokaryotic origin, most likely due to horizontal transfer.

Transcriptome studies of mosquito responses to parasite infection complement those done with whole-genome or selected microarrays. Sequencing of 1485 random clones obtained from subtracted cDNA libraries of *An. stephensi* infected with *P. berghei* identified >1100 unique reads (Srinivasan *et al.*, 2004). These provided information on both mosquito and parasite genes that were regulated during infection. For example, a mosquito gene encoding a fibrinogen domain was induced coincidentally with the parasite transition from ookinete to oocyst. Using the same approach, Ancaspase-7, an *Anopheles* effector caspase activated during *Plasmodium* invasion of midgut was discovered (Abraham *et al.*, 2004). A similar approach was used to identify genes in *Ae. aegypti* midguts expressed differentially in mosquitoes susceptible or refractory to the avian malaria parasite *P. gallinaceum* (Chen *et al.*, 2004). Approximately 2.3% of 1200 midgut cDNA were expressed differentially between the susceptible and refractory mosquito populations. Of particular note, four genes corresponded to marker genes used in quantitative-trait analysis of parasite refractoriness in the same mosquito (Severson *et al.*, 2002).

III. CONCLUDING REMARKS

Recent years have witnessed rapid progress in the development of new techniques to study gene expression in mosquitoes. Development of transgenesis and a growing complement of effector molecules bring closer the promise of the use of a genetic strategy to control the transmission of mosquito-borne diseases. The expression of fluorescent proteins facilitates the screening of transformants and functional analysis of *cis*-acting regulatory regions of genes. However, the expression levels of heterologous genes in transgenic mosquitoes are usually low, which might affect their utility in genes designed to block pathogen transmission. Identification and characterization of more robust gene promoters are required to overcome these low expression levels. Also, more tissue-specific gene promoters are required to express multiple effector molecules in the same transgenic mosquito, which is expected to increase the effect of antipathogen phenotype as well as decrease the possibility of development of pathogen resistance to the effectors. There is yet no complete inhibition of pathogen transmission in transgenic mosquitoes.

Rapid progress has been seen in the field of genomics due to the availability of three mosquito genomes. Genes differentially expressed following blood meals, parasite infection and as a consequence of insecticide resistance have been studied extensively. Remarkable expansions of major gene families involved in odor perception, immune responses, and salivary gland products provide materials for years of future research. Both the genomics and transcriptome studies have been complemented immensely by studies using RNAi for gene silencing. RNAi allows characterization of genes *in vivo* which can later be targeted for transmission blocking studies. It also helps in understanding the mechanism of gene regulation.

The field of mosquito molecular biology would benefit from the sequencing of additional mosquito genomes. Population-level studies of gene variation also could provide insights into aspects of behavior that make these insect such dangerous animals. Practically, better transgenesis tools are needed if the techniques are ever to see the widespread applications that are characteristic of research with *D. melanogaster*. Methods for long-term storage of strains also are needed. Finally, the field would be strengthened greatly by the recruitment of new, young and creative researchers looking for topics in translational science.

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Figure 2.1.

Expression of marker and reporter transgenes in the malaria vector mosquito, *Anopheles stephensi*. (A) Fourth-instar larva under ambient light, (B) Same larva as in (A) seen under fluorescence microscopy. Larval eyes (*e*), nervous tissue (*nt*), and anal papillae (*ap*) fluoresce with DsRed. (C) Adult females expressing the cyan fluorescent protein gene controlled by the *AsVg1* promoter (see Nirmala *et al.*, 2006). Specific fluorescence is detected in fat body tissues in the thorax and abdomen. Images courtesy of J. M. Sandoval.

Table 2.1**Mosquito Species That Have Been Transformed**

Mosquito species	Transposon	References
<i>Aedes aegypti</i>	<i>Hermes</i> ^a	Jasinskiene <i>et al.</i> (1998)
	<i>Mos1 (mariner)</i> ^{a,b}	Coates <i>et al.</i> (1998 (2000)
	<i>piggyBac</i> ^a	Kokoza <i>et al.</i> (2001a)
<i>Ae. fluviatilis</i>	<i>piggyBac</i>	Rodrigues <i>et al.</i> (2008)
<i>Anopheles gambiae</i>	<i>piggyBac</i> ^a	Grossman <i>et al.</i> (2001)
<i>An. stephensi</i>	<i>Minos</i> ^a	Catteruccia <i>et al.</i> (2000)
	<i>piggyBac</i> ^a	Nolan <i>et al.</i> (2002)
<i>An. albimanus</i>	<i>piggyBac</i> ^a	Perera <i>et al.</i> (2002)
<i>Culex quinquefasciatus</i>	<i>Hermes</i>	Allen <i>et al.</i> (2001)

^a Mobility assay data published.

^b Purified *Mos1 (mariner)* transposase also successful.

Table 2.2

Transposons Used in Mosquito Transgenesis

Transposon	Family	Origin	~Size (kb) ^a	ITR ^b length	Target site	References
<i>Hermes</i>	<i>hAT</i>	<i>Musca domestica</i>	2.7	17	GTNCAGAC	O'Brochia <i>et al.</i> (1996)
<i>Mos1</i>	<i>Tc1-mariner</i>	<i>Drosophila mauritiana</i>	1.3	~30	TA	Medhora <i>et al.</i> (1991)
<i>Minos</i>	<i>Tc1-mariner</i>	<i>Drosophila hydei</i>	1.4	254	TA	Franz and Savakis (1991) and Loukeris <i>et al.</i> (1995)
<i>piggyBac</i>	Novel	Lepidopteran baculovirus	2.5	13	TTAA	Fraser <i>et al.</i> (1996)
<i>Th5</i>	<i>IS50</i>	Enteric bacteria	5.8	19	9 bp, variable	Berg <i>et al.</i> (1983)

^a Approximate size in kilobases.

^b Inverted terminal repeat length in nucleotides.

Table 2.3

Functional Control DNA in Mosquitoes

Transgene		Expression profile ^a			
Promoter	Reporter	Species	Sex	Tissue	Temporal
<i>Pax6</i>	EGFP, DsRed	<i>Ae. aegypti</i> , <i>An. stephensi</i>	Male/female	Eyes	Constitutive
<i>AeVg</i>	DefA	<i>Ae. aegypti</i>	Female	Fat body	Blood meal inducible
<i>AeVg1</i>	N2ScFv	<i>Ae. aegypti</i>	Female	Fat body	Blood meal inducible
<i>AeVgC</i>	Luciferase	<i>Ae. aegypti</i>	Female	Fat body	Blood meal inducible
<i>AgVgT2</i>	EGFP	<i>An. stephensi</i>	Female	Fat body	Blood meal inducible
<i>AaVgR</i>	DsRed	<i>Ae. aegypti</i>	Female	Ovary	Blood meal inducible
<i>AsVg1</i>	CFP	<i>An. stephensi</i>	Female	Fat body	Blood meal inducible
<i>DmUb</i>	N2ScFv	<i>Ae. aegypti</i>	ND ^b	ND ^b	Constitutive
<i>β2-tub</i>	EGFP	<i>An. stephensi</i> , <i>Ae. aegypti</i>	Male	Gonads	Constitutive
<i>AeCP</i>	Luciferase	<i>Ae. aegypti</i>	Female	Midgut	Blood meal inducible
<i>AeCP</i>	CecA	<i>An. gambiae</i>	Female	Midgut	Blood meal inducible
<i>AgCP</i>	Luciferase	<i>Ae. aegypti</i>	Female	Midgut	Blood meal inducible
<i>AgCP</i>	SM1, PLA2	<i>An. stephensi</i>	Female	Midgut	Blood meal inducible
<i>AgAper1</i>	Mutated PLA2	<i>Ae. fluviatilis</i>	Female	Midgut	Blood meal inducible
<i>AgAper1</i>	PLA2	<i>An. stephensi</i>	Female	Midgut	Blood meal inducible
<i>act88F</i>	EGFP	<i>Cx. quinque</i>	Male/female	Muscle	Constitutive
<i>actin</i>	EGFP	<i>An. stephensi</i> , <i>Cx. quinque</i>	Male/female	Muscle	Constitutive
<i>actin5C</i>	EGFP	<i>Ae. aegypti</i>	Male/female	All tissue	Constitutive
<i>AAPP</i>	DsRed	<i>An. stephensi</i>	Female	Salivary glands	Blood meal inducible
<i>Apy</i>	Luciferase	<i>Ae. aegypti</i>	Female	Salivary glands	Constitutive
<i>Mall</i>	Luciferase	<i>Ae. aegypti</i>	Female	Salivary glands	Constitutive
<i>Hsp70</i>	Luciferase	<i>Ae. aegypti</i>	Female	Salivary glands	Adult
<i>Rdl</i>	Luciferase	<i>Ae. aegypti</i>	Male/female	ND ^b	Embryo
<i>Ferritin LCH</i>	Luciferase	<i>Ae. aegypti</i>	ND ^b	ND ^b	Cell culture

Catteruccia *et al.* (2005) and Nimmo *et al.* (2006)

Kokoza *et al.* (2000, 2001b)

Jasinskiene *et al.* (2007)

Iscoe *et al.* (2007)

Chen *et al.* (2007)

Cho *et al.* (2006)

Nirmala *et al.* (2006)

Jasinskiene *et al.* (2007)

Catteruccia *et al.* (2005) and smith *et al.* (2007)

Moreira *et al.* (2000)

Kim *et al.* (2004)

Moreira *et al.* (2000)

Ito *et al.* (2002) and Moreira *et al.* (2002)

Rodrigues *et al.* (2008)

Abraham *et al.* (2005)

Allen and Christensen (2004)

Allen *et al.* (2001) and Catteruccia *et al.* (2000, 2003)

Pinkerton *et al.* (2000)

Yoshida and Watanabe (2006)

Coates *et al.* (1999)

Coates *et al.* (1999)

Morris *et al.* (1995)

Shotkoski *et al.* (1996)

Pham and Chavez (2005)

Transgene			Expression profile ^a			
Promoter	Reporter	Species	Sex	Tissue	Temporal	Reference
<i>CecB</i>	Luciferase	<i>Ae. aegypti</i>	Female	Fat body	Lipopolysaccharide inducible	Isoe <i>et al.</i> (2007)
<i>nanos</i>	<i>Mos1</i> transposase	<i>Ae. aegypti</i>	Female	Ovaries/embryos	Embryo	Adelman <i>et al.</i> (2007)
<i>AgApy</i>	<i>LacZ</i>	<i>An. stephensi</i>	Female/male	Salivary glands	Constitutive	Lombardo <i>et al.</i> (2005)

^aThese columns indicate the species in which the DNA was tested and summary information on the expression profile.

^bNot determined.