Gal4-based Enhancer-Trapping in the Malaria Mosquito Anopheles stephensi

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ABSTRACT Transposon-based forward and reverse genetic technologies will contribute greatly to ongoing efforts to study mosquito functional genomics. A piggyBac transposon-based enhancer-trap system was developed that functions efficiently in the human malaria vector, Anopheles stephensi. The system consists of six transgenic lines of Anopheles stephensi, each with a single piggyBac-Gal4 element in a unique genomic location; six lines with a single piggyBac-UAS tdTomato element; and two lines, each with a single Minos element containing the piggyBac-transposase gene under the regulatory control of the hsp70 promoter from Drosophila melanogaster. Enhancer detection depended upon the efficient remobilization of piggyBac-Gal4 transposons, which contain the yeast transcription factor gene Gal4 under the regulatory control of a basal promoter. Gal4 expression was detected through the expression of the fluorescent protein gene tdTomato under the regulatory control of a promoter with Gal4-binding UAS elements. From five genetic screens for larval- and adult-specific enhancers, 314 progeny were recovered from 24,250 total progeny (1.3%) with unique patterns of tdTomato expression arising from the influence of an enhancer. The frequency of piggyBac remobilization and enhancer detection was 2.5- to 3-fold higher in female germ lines compared with male germ lines. A small collection of enhancer-trap lines are described in which Gal4 expression occurred in adult female salivary glands, midgut, and fat body, either singly or in combination. These three tissues play critical roles during the infection of Anopheles stephensi by malaria-causing Plasmodium parasites. This system and the lines generated using it will be valuable resources to ongoing mosquito functional genomics efforts.

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Vector-borne diseases, such as mosquito-transmitted malaria, dengue fever, and filariasis, among many others, not only remain health threats to a significant fraction of the world’s population but also significantly impact the economies of countries in which there is intense transmission (World Health Organization 2010). In the case of malaria, controlling the mosquito vectors of malaria-causing Plasmodium parasites continues to be a major component of malaria control efforts. Understanding the genetic and molecular genetic basis of insecticide resistance, olfaction, reproductive physiology and the immune system of Anopheles mosquitoes figures heavily into contemporary ideas for developing new strategies for controlling mosquito populations and Plasmodium transmission (Carey et al. 2010; Catteruccia 2007; Enayati and Hemingway 2011; Alonso et al. 2011).

Recent advances in mosquito molecular genetics have depended upon the availability of whole-genome sequence data and a host of technological advances, including transcription-profiling and RNA-based gene-silencing technologies (Blandin et al. 2002; Dimopoulos et al. 2000; Holt et al. 2002). However, powerful functional genomics technologies for finding and mutating mosquito genes as well as regulating transgene expression, such as enhancer- and gene-trap technologies, have been lacking.

Transposons can be used as platforms upon which some of these powerful functional genomics technologies can be constructed. Transposon-based enhancer detection is an effective way to sense the presence of enhancers and when coupled to robust binary transcription...
regulatory systems such as the Gal4 system, the “trapped” enhancers can be used to regulate the expression of any transgene under the regulatory control of a promoter containing Gal4 upstream activation sequences (UAS) without having to physically isolate and characterize the regulatory elements (Brand and Perrimon 1993). Gene traps enable genes to be detected based on the patterns of expression of transgenes carried on the transposon, and in many cases, transposon integration results in disabling the target gene (Stanford et al. 2001). The resulting recessive hypomorph or null mutations can be of great value in efforts to determine a gene’s function. The power of these technologies and the myriad variations that exist are particularly well displayed in many studies of the popular animal model systems Drosophila melanogaster and Mus musculus (Bellen 1999; Duffy 2002; Friedel and Soriano 2010) and to a lesser extent in “nonmodel” systems (Awazu et al. 2004, 2007; Balcíunas et al. 2004; Kontarakis et al. 2011; Lorenzen et al. 2007; Trauner et al. 2009; Uchino et al. 2008). Vector biologists could benefit substantially from the availability of these technologies for the study of mosquitoes.

Transposon-based transgenic technologies have been available for mosquitoes for over a decade but they are utilized somewhat infrequently because the creation of primary transgenic mosquitoes can be technically challenging and because some transposons, once integrated, have shown little or no remobilization activity, severely limiting their utility as functional genomics tools. In Aedes aegypti, the transposons Hermes, Mos1, and piggyBac, although effective as vectors for creating transgenic mosquitoes, cannot be remobilized or are remobilized rarely in the presence of functional transposase following their integration into the genome of this species (O’Brochta et al. 2004; Sutharam et al. 2007; Wilson et al. 2003). Similar observations were made in Anopheles stephensi concerning the Minos transposon (Scali et al. 2007). Consequently, vector biologists have been unable to develop powerful transposon-based gene-finding and analysis technologies. Fortunately, the remobilization behavior of piggyBac elements integrated into the genome of Anopheles stephensi is quite different from that of Minos; piggyBac is highly active in An. stephensi in the presence of transposase, permitting the development of a variety of much-needed gene-finding and analysis technologies in this species (O’Brochta et al. 2011).

Here we report on the creation and performance of a Gal4-based enhancer–trap system for An. stephensi. We show that enhancers are readily detected with our system and that this technology can be used to create lines of mosquitoes with patterns of Gal4 expression particularly useful for regulating the expression of transgenes in cells and tissues relevant to the study of mosquito/parasite interactions.

**MATERIAL AND METHODS**

**Mosquitoes**

Anopheles stephensi were grown at 29°C (80% relative humidity for adults), and larvae were provided with pulverized fish food (TetraMin Tropical Flakes) *ad libitum*, while adults were provided with 10% sucrose continuously. Adult females were occasionally allowed to feed on adult mice to obtain a blood meal, which was necessary for reproduction. The use of mice was with the approval and oversight of the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland, College Park, operating under the National Institutes of Health’s Office of Laboratory Animal Welfare guidelines. Mosquito blood-feeding protocols involving mice were not terminal, and animal pain and distress were minimized with the use of anesthetics with the approval of the IACUC.

**SDA 500:** This is a wild-type strain of An. stephensi originally isolated in Pakistan and selected in the laboratory for susceptibility to Plasmodium falciparum infection (Feldmann et al. 1990).

**UMITF-PB-F2DsRed and UMITF-PB-M5DsRed:** These are transgenic lines of SDA 500, with each line containing a single copy of the Minos gene vector from pMi[3xP3-DsRed]-hsp70-piggyBac (Horn et al. 2003; O’Brochta et al. 2011) (Figure 1). This vector contains the piggyBac transposase open reading frame (ORF) under the regulatory control of the promoter from the hsp70 gene from *D. melanogaster* (Horn et al. 2003). Heat-shock induction was not necessary for expression of piggyBac transposase in the germ line or soma of these mosquitoes (O’Brochta et al. 2011). We refer here to lines UMITF-PB-F2DsRed and UMITF-PB-M5DsRed as F2 and M5, respectively.

**Vectors**

**PB-GAL4:** This is a piggyBac vector with 329 bp of the 5’ terminal sequences and 690 bp of the 3’ terminal sequences of piggyBac containing the Gal4 ORF under the regulatory control of the piggyBac transposase gene’s promoter in addition to a visible marker gene encoding the enhanced cyan fluorescent protein (ECFP) under the regulatory control of the 3xP3 promoter (Berghammer et al. 1999). This vector was constructed using Gateway recombination cloning technology (Invitrogen, Grand Island, NY), in which four recombination modules were simultaneously recombined into a destination plasmid. The first module consisted of the first 329 bp of the 5’ terminal sequences of piggyBac (GenBank J04364). The second module consisted of the Gal4 ORF from pGaTB attached to the 3’ UTR of the hsp70 gene of *D. melanogaster* (Brand and Perrimon 1993). When the first and second modules were joined during site-specific recombination, the piggyBac transposase promoter was juxtaposed to the Gal4 ORF. The third module consisted of ECFP under the regulatory control of the 3xP3 promoter, which was isolated from pXL-pBac-ECFP (Berghammer et al. 1999; Li et al. 2005). Recombination between modules two and three joined the Gal4 enhancer detector module and the marker gene such that transcription of each was in opposite directions. The fourth module consisted of last 690 bp of the 3’ terminal sequences of piggyBac (Figure 1).

**Figure 1** Organization of piggyBac and Minos vectors. PB-GAL4 has the Gal4 ORF (“Gal4”) located just 3’ of the promoter for the piggyBac transposase. piggyBac sequences containing the 5’ and 3’ inverted terminal repeats and sub-terminal sequences are shown (black arrows; “5’PB” and “3’PB”). This element contains the ECFP gene under the regulatory control of a central nervous tissue-specific promoter (“3xP3ECFP”). PB-UAStTomato contains the inverted repeats and sub-terminal sequences of piggyBac (black arrows; “5’PB” and “3’PB”), the EYPF gene under the regulatory control of a central nervous tissue-specific promoter (“3xP3EYFP”) and the ORF of tdTomato under the regulatory control of a minimal promoter with five optimized GAL4 binding sites (“UAStTomato”) (Brand and Perrimon 1993). pMi[3xP3-DsRed]-hsp70-piggyBac is based on the description in Horn et al. (2003) and contains the 5’ and 3’ inverted terminal repeats and sub-terminal sequences of Minos (black arrows; “5’Mi” and “3’Mi”). This element contains the DsRed gene under the regulatory control of a central nervous tissue-specific promoter (“3xP3DsRed”), and the piggyBac transposase ORF under the regulatory control of the hsp70 promoter from *D. melanogaster* (hsp70Ptransposase). Dotted lines with arrows show the direction of transcription associated with all transgenes.
**PB-UAS*tdTomato**: This is a piggyBac vector with 671 bp of the 5’ terminal sequences and 690 bp of the 3’ terminal sequences of piggyBac containing the tandem-dimer form of the *DsRed* variant *Tomato* (*tdTomato*) (Shaner et al. 2004) under the regulatory control of a promoter with Gal4-binding and upstream activating sequences (UAS), along with a marker gene consisting of the enhanced yellow fluorescent protein (EYFP) under the regulatory control of the 3xP3 promoter. This vector was also constructed using Gateway recombination cloning technology (Life Technologies, Grand Island, NY) involving the simultaneous recombination of four recombination modules into a destination plasmid. The first module contained 690 bp of the 3’ terminal sequences of piggyBac. The second module contained 1.5 kb of the *tdTomato* ORF from ptdTomato (Clontech, Mountain View, CA) inserted into pUAST-atB at the EcoRI/NotI sites between the promoter region containing five UAS elements and the 3’ UTR of *hsp70* from *D. melanogaster*. The third module contained EYFP under the regulatory control of the 3xP3 promoter, and the fourth module contained 671 bp of the 5’ terminal sequences of piggyBac (Figure 1).

**Mosquito transformation**

Transgenic *An. stephensi* were created in the University of Maryland, College Park, Institute for Bioscience and Biotechnology Research’s Insect Transformation Facility (http://www.ibbr.umd.edu/facilities/if) by injecting preblastoderm embryos of SDA 500 *An. stephensi* with vector-containing plasmids and plasmids expressing piggyBac transposase (phsp-PBac) (Handler and Harrell 1999). Vectors and transposase-expressing plasmids were each at 50 ng/μl in microinjection buffer (5 mM KCl, 0.1 mM NaPO4; pH 6.8). Insects developing from injected embryos and surviving to adulthood were pooled according to sex and mated to noninjected SDA 500 adults of the opposite sex. The progeny were screened as larvae for the expression of ECFP or EYFP, and transgenic individuals were used to establish lines. The piggyBac insertion sites were determined using splinkerette-PCR after lines were established (see below), and the DNA sequence of their integration sites were deposited in GenBank (accession numbers JX242566–JX242578)

**Gal4 remobilization crosses and enhancer detection**

Approximately 100 PB-Gal4–containing individuals (male or female, depending on the cross) were mated *en masse* with ~100 piggyBac transposase-expressing individuals of the opposite sex (*UMITF-PB-M5*ΔNot and *UMITF-PB-F2*ΔNot). Approximately 100 individuals heterozygous for both PB-Gal4 and *UMITF-PB-F2*ΔRed or *UMITF-PB-M5*ΔRed were mated to ~100 PB-UAS*tdTomato* individuals *en masse*, and the resulting progeny were screened as third or fourth instar larvae and as adults for *tdTomato* expression. Although piggyBac transposase was under the regulatory control of the promoter from the *hsp70* gene from *D. melanogaster*, heterozygous individuals containing both PB-Gal4 and piggyBac transposase were not heat-shocked. Earlier work showed that heat shock was unnecessary for transposase expression and piggyBac remobilization using these and similar lines (O’Brochta et al. 2011). The number of individuals with novel patterns of *tdTomato* expression was recorded, and selected individuals were used to start lines.

**Splinkerette-PCR**

The splinkerette-PCR genotyping method is based on amplification of genomic DNA containing the 5’ or 3’ end of the piggyBac element and a variable amount of adjoining genomic DNA (Devon et al. 1995; Potter and Luo 2010). This method was used to confirm the integration of piggyBac into the genome, to compare genotypes of transgenic individuals, and to sequence the genomic DNA flanking integrated piggyBac elements to locate the integration site within the genome. Splinkerette-PCR was performed as described previously using genomic DNA isolated from individual third or fourth instar larvae or adults (O’Brochta et al. 2011; Potter and Luo 2010).

**Bioinformatics analysis**

DNA sequence data obtained from splinkerette-PCR, representing genomic DNA flanking the piggyBac enhancer-trap element, was used to query publicly available *An. gambiae* genome sequence data and an assembled draft genome of *An. stephensi* [created and made available by Dr. Zhijian (Jake) Tu at Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, and now publicly available on VectorBase (Lawson et al. 2007)]. Insertion sites were located to scaffolds within the current *An. stephensi* genome release, AstEv1. All DNA sequence queries were performed using the algorithmic basic local alignment search tool (BLAST) (Altschul et al. 1990).

**Microscopy**

The patterns of *tdTomato* expression were determined by microscopic observations of larvae, pupae, and adults using an Olympus MXV10 fluorescent dissecting microscope equipped with Chroma filters (Chroma Technology Corporation, Bellows Falls, VT) 49001 ET-CFP (excitation, 436/20; emission, 480/40; dichroic, 455), 49002 ET-GFP (excitation, 470/40; emission, 525/50; dichroic, 495), 49003 ET-EYFP (excitation, 500/20; emission, 535/50; dichroic, 515), 49005 ET-DSRed (excitation, 545/30; emission, 620/60; dichroic, 570) as well as a Zeiss Axiom Imager A1 fluorescent compound microscope with Zeiss filter set 20 (excitation, 546/12; emission, 575–640; dichroic, 560) and filter set 38HE (excitation, 470/40; emission, 525/30; dichroic, 495).

**RESULTS**

**Transgenic lines**

Six independent Gal4 enhancer-trap–containing lines were created, each with a single piggyBac element. Similarly, six UAS*tdTomato*-containing lines were created, each of which contained a single UAS*tdTomato* transgene (Table 1). The locations of the inserted elements varied, and all integrations involved canonical cut-and-paste transposition into TTAA target sites, as expected when using piggyBac transposons (Fraser 2000). The chromosomal locations of integrated elements in *An. stephensi* were assigned to scaffolds in the

| Table 1 Enhancer-trap system for *Anopheles stephensi* |
|-----------------|-----------------|-----------------|
| **Line**        | **Location**    | **GenBank**     |
| UMITF-PB-Gal4.1 | 04796: 42751-54 | JX242568        |
| UMITF-PB-Gal4.2 | 05657: 162381-84 | JX242569        |
| UMITF-PB-Gal4.3 | 03905: 171549-52 | JX242570        |
| UMITF-PB-Gal4.4 | 03863: 483-86   | JX242571        |
| UMITF-PB-Gal4.5 | ND              |                 |
| UMITF-PB-Gal4.6 | 01707: 601922-25 | JX242572        |
| UMITF-UAS:tdT1  | 02731: 149811-14 | JX242573        |
| UMITF-UAS:tdT2  | 01636: 9998-01  | JX242574        |
| UMITF-UAS:tdT3  | 02729: 107840-43 | JX242575        |
| UMITF-UAS:tdT4  | 04375: 250069-72 | JX242576        |
| UMITF-UAS:tdT6  | 05523: 17863-66  | JX242577         |
| UMITF-UAS:tdT8  | 00733: 188438-41 | JX242578        |
| UMITF-PB-F2*ΔRed| 01724: 355056-57 | JX242566        |
| UMITF-PB-M5*ΔRed| 02306: 81725-26  | JX242567        |

*ND*, not determined (i.e. no splinkerette data were obtained).

The scaffold number in *An. stephensi* genome release AstEv1 in VectorBase (Lawson et al. 2007) is followed by the nucleotide coordinates of the TTAA (piggyBac) or TA (Mnas) target sites within that scaffold.

GenBank accession numbers.
most current An. stephensi genome-release, AsteV1, available on VectorBase (Lawson et al. 2007) (Table 1). None of the PBGal4-containing lines, with the exception of UMITF-PBGal4.5, had detectable Gal4 expression and were therefore sensitive reporters of enhancers encountered during element remobilization. Line UMITF-PBGal4.5 had low levels of Gal4 expression in the central nervous system, including the brain and ventral ganglia, due to the presence of an enhancer near the primary integration site. This element can still be used for enhancer-trap screens, depending on the target phenotypes that are of interest in the screen. None of the UAStdTomato-containing lines had detectable tdTomato gene expression in the absence of Gal4.

**Frequency of enhancer detection**

We screened a total 24,250 larvae and adult progeny for the presence of remobilized Gal4 enhancer-trap elements resulting in the expression of UAStdTomato. These progeny were obtained from five independent crosses involving the use of both piggyBac-transposase–expressing lines M5 and F2 (Table 2). As observed in an earlier study, a 2.5- to 3-fold higher rate of piggyBac remobilization (enhancer-trap events) was observed in the germ line of females compared with the germ line of males (Table 2) (O’Brochta et al. 2011). Overall, the frequency of enhancer detection was approximately one enhancer-trap event per 51 progeny screened (2%) when remobilization occurred in the germ line of females. When remobilization occurred in the germ line of males, the frequency of enhancer detection was approximately one enhancer-trap event per 130 progeny screened (0.8%). We did not observe any significant difference between the remobilization frequencies observed when the two piggyBac-transposase–expressing lines F2 and M5 were used ($z = -2.4; P = 0.0022$). When enhancer-trap events were detected, they were almost always represented by a single individual among the progeny. Of the 317 progeny with tdTomato expression, we estimate that most resulted from independent transposition events.

**Somatic activity**

The transposase–expressing lines F2 and M5 both expressed piggyBac transposase under the regulatory control of the hsp70 promoter from D. melanogaster, and consequently, piggyBac remobilization was not expected to be confined to the germ line of insects containing both a Gal4 enhancer-trap element and piggyBac transposase. Indeed, in the F1 heterozygotes containing a Gal4 enhancer-trap element and a piggyBac transposase-expressing transgene, we observed clear evidence of somatic movement of the Gal4 enhancer-trap element (Figure 2).

When the piggyBac transposase–expressing transgene originated from the F2 line, the F1 heterozygotes displayed irregular patterns of tdTomato expression involving small patches of cells, giving the larvae and adults a distinctly mottled appearance (Figure 2A). These patterns were asymmetrical and not heritable, which is consistent with their somatic nature. When the piggyBac–transposase–expressing transgene originated from the M5 line, F1 heterozygotes frequently showed expression of tdTomato in individual muscles or groups of muscles in larvae (Figure 2B). The patterns of tdTomato expression in the muscles of F1 heterozygotes were also always asymmetrical, and we attribute these patterns to the presence of a muscle-specific enhancer influencing the somatic expression of the hsp70-regulated transposase transgene in line M5. We speculate that this results in elevated levels of piggyBac transposase in muscle cells, thereby increasing the frequency of remobilization of the Gal4 enhancer-trap element in these cells and, consequently, the probability of observing tdTomato expression.

**Germ line activity**

Outcrossing F1 heterozygotes with individuals homozygous for a UAStdTomato-containing transgene resulted in the detection of 317 progeny with tdTomato expression patterns, consistent with the detection of an enhancer by the Gal4 enhancer-trap element. Some of these individuals were retained and used to establish permanent lines so they could be used in the future for regulating transgene expression. We describe some of those lines here.

**UMITF-C2F8:** Gal4 is expressed strongly in the abdomen of larvae, including the fat body and a distinct region of the posterior midgut (Figure 3A). In this line, the enhancer is not only regulating expression of Gal4 but also the ECFP marker gene that is under the regulatory control of the nerve-specific 3xP3 promoter. Although the 3xP3 promoter is known to be sensitive to enhancers, tdTomato expression and ECFP expression did not always overlap (see line UMITF-C2F4 below) (O’Brochta et al. 2011; Trauner et al. 2009). Gal4 in this line was expressed in adult males and females (in Figure 3, compare B–E with F–I). In both sexes, strong Gal4 expression was seen in the halteres (Figure 3, B and F). In females, aside from the halteres, Gal4 expression was only detected in the posterior midgut both before and after blood feeding (Figure 3, C–E). No Gal4 expression was detected in any other region of the alimentary canal, ovaries, or carcass. In adult males, the alimentary canal did not show any Gal4 expression (Figure 3, G and H), although there was some expression associated with the abdominal epidermis (Figure 3F). Males also showed strong expression in the maxillary palps (Figure 3I).

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**Table 2 Gal4/UAS-based enhancer-trap screens in Anopheles stephensi**

| Cross     | F1♂♂ | F1♀♀ | Screened | tdTomato | Percent |
|-----------|-------|-------|----------|-----------|---------|
| A         | PBGal4.1 / M5  | UAS:tdT1 | 4700 | 26 | 0.553*  |
| B         | UAS:tdT1 | PBGal4.1 / M5 | 5500 | 92 | 1.673*  |
| C         | PBGal4.1 / F2 | UAS:tdT1 | 5500 | 52 | 0.945*  |
| D         | UAS:tdT1 | PBGal4.1 / F2 | 4450 | 102 | 2.292*  |
| E         | UAS:tdT2 | PBGal4.1 / M5 | 4100 | 45 | 1.098   |

| Totals    | 24250 | 317   | 1.307    |

* M5 and F2 refer to piggyBac transposase–expressing lines UMITF-PB-M5 and UMITF-PB-F2, respectively. All other lines designations omit the UMITF prefix.

b Only insects with PBGal4 (3xP3ECFP) and UAS:tdT (3xP3YFP) were counted. Screens were conducted at fourth instar and adult stages.

c Total number of fourth instar larvae or adults expressing tdTomato.

d (Number of tdTomato-expressing insects ÷ Total number of larvae screened) × 100.

e Proportion expressing tdTomato in Crosses A and B were significantly different. z = -5.27, P < 0.001.

f Proportion expressing tdTomato in Crosses C and D were significantly different. z = -5.41, P < 0.001.
UMITF-2MCL14: This line has widespread Gal4 expression in both the larval and adult stages (Figure 4). In late instar larvae, Gal4 expression occurs in some major muscle groups in the head, thorax, and abdomen, including muscles involved in moving mouthparts and longitudinal muscles extending down the ventral surface of the larva (Figure 4, A and B). Gal4 expression is also seen in the ventral ganglia of the larva, in or around the salivary glands, and in the larval antenna (Figure 4, A and B). In adult males and females, widespread Gal4 expression is seen in what appears to be neuronal tissue in the antennae, maxillary palps, and legs (Figure 4, C and D). The anterior and posterior midguts of unfed females express Gal4 (Figure 4, E and I), as do cells of the crop (Figure 4, F and G) and previtellogenic ovaries (Figure 4H).

UMITF-C2F81: Late instar larvae have Gal4 expression in the salivary glands and some neuronal tissue, including the ventral ganglia, brain, and lateral structures that appear to correspond to neurohemal organs (Figure 5, A–C). In this line, expression of the 3xP3ECFP marker gene is not influenced by the enhancer responsible for determining the observed pattern of Gal4 as indicated by expression of tdTomato but not ECFP in the salivary glands (Figure 5B). In adults, both males and females have Gal4 expression in the salivary glands and brain (Figure 5, D and E). The salivary glands of females have Gal4 expression in the lateral and medial lobes (Figure 5E).

UMITF-2MCL6: Gal4 is expressed in the larval salivary glands, cells at the base of larval setae, and cells in the main trunk of the tracheal system (Figure 6, A and B). Adults have Gal4 expression in cells at the base of all scales and sensory bristles throughout the body (Figure 6, D–F). In addition, Gal4 is expressed in the lateral lobes of the salivary glands of adult females, although there is no Gal4 expression in the medial lobe (Figure 6, C, G, and H).

UMITF-AEA1: No Gal4 expression was detectable in the larval stages of this line. In adult females, Gal4 expression was detected in abdominal fat body and weakly in the salivary glands (Figure 7A). Gal4 was also expressed specifically in the pedicel at the base of the antenna of adults (Figure 7B).

UMITF-MBL24: Larvae of this line have Gal4 expression in the salivary glands, posterior midgut, and the abdominal fat body,

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**Figure 2** Somatic activity of enhancer-trap elements. (A) Fourth instar larva heterozygous for a Gal4 enhancer-trap element and the transposase-expressing transgene from the F2 line. The mottled appearance is due to somatic clones of cells (arrows) in which somatic movement of the enhancer-trap element resulted in enhanced expression in subpopulations of larval cells. (B) Fourth instar larva heterozygous for a Gal4 enhancer-trap element and the transposase-expressing transgene from the M5 line. Enhanced Gal4 expression was often seen in individual muscles or groups of muscle in asymmetrical patterns that were not heritable, indicating that these were somatic clones. Frequent enhancement of tdTomato expression in muscle cells was likely due to the presence of a muscle-specific enhancer near the piggyBac transposase-containing transgene in the M5 line, resulting in elevated levels of transposition of the Gal4 enhancer-trap element in these cells.

**Figure 3** Line UMITF-C2F8. (A) Whole fourth-instar larva and dissected midgut. Arrows point to fat body and an intense region of tdTomato expression in the midgut. Dissected midgut shows overlapping patterns of expression of the 3xP3ECFP marker gene associated with the Gal4-containing piggyBac element and the UASldTomato transgene. The enhancer influencing Gal4 expression is also having a similar effect on 3xP3ECFP. (B) Ventral view of an unfed adult female. Arrows point to tdTomato expression in the abdomen and the distal region of the halteres. (C) Dissected alimentary canal of an unfed female. a-mg, anterior midgut; p-mg, posterior midgut (arrow); mt, Malpighian tubules; ov, ovaries. (D) Same alimentary canal as in (C) showing tdTomato expression only in the posterior midgut. (E) Close-up of the posterior midgut shown in (D). (F) Ventral view of an adult male with strong expression in the halteres (arrow) and abdomen. (G) Dissected alimentary canal of a male showing no tdTomato expression. (I) Maxillary palps from a male with strong tdTomato expression present at the terminal region.
The patterns of Gal4 expression in adult males and females strongly parallel the patterns of Gal4 expression observed in the larval stages (Figure 8B and C). The lateral and medial lobes of the adult female salivary glands strongly express Gal4 (Figure 8C). In the midgut of both fed and unfed females, strong Gal4 expression is observed specifically in the posterior midgut (Figure 8C–F). The fat body in the female abdomen also strongly expresses Gal4 (Figure 8C and G).

UMITF-MDL8: Gal4 is expressed throughout larvae, including muscles, fat body, and nervous tissue (Figure 9A). Gal4 expression in the larval midgut is concentrated anteriorly in the caecae and in the posterior region of the midgut (Figure 9B–D). Strong expression is also seen in the Malpighian tubules (Figure 9B and D). Likewise in adult males and females, Gal4 expression is widespread (Figure 9, E–G). Adult males and females have Gal4 expression throughout the nervous system, including antennae, maxillary palps, legs, and brain (Figure 9, E–G). In adult females, all lobes of the salivary glands are expressing Gal4, as are the ovaries (Figure 9, H and I). Gal4 expression is also seen in the alimentary canal, beginning with the cardia and including the anterior and posterior midgut and the Malpighian tubules (Figure 9J and K). Gal4 expression in the adult female midgut is not uniform, with distinctly more expression in the anterior midgut and cardia, as well as the posterior half of the posterior midgut (Figure 9K). Gal4 expression in the adult female midgut is feeding independent (Figure 9L).
In both larvae and adults, Gal4 expression is strongly localized to the salivary glands of larvae and in no other cells of the larva except for scattered stellate cells in the abdominal epidermis (Figure 11, A and B). In adults, Gal4 expression is not observed in the salivary glands (Figure 11C), but there are isolated and evenly distributed cells in or just below the epidermis of the abdomen that express Gal4 (Figure 11D).

UMITF-2C2M8: Gal4 expression in this line is restricted to only the salivary glands of larvae (Figure 10A). Gal4 expression could not be detected in any other tissue in adult male or female mosquitoes (Figure 10B).

UMITF-DEA9A: Gal4 expression is strongly localized to the salivary glands of larvae and in no other cells of the larva except for scattered stellate cells in the abdominal epidermis (Figure 11, A and B). In adults, Gal4 expression is distinctly absent from the central nervous system, including the brain and ventral ganglia (Figure 12, B and C). A similar distribution of Gal4 expression is seen in adults, with cells at the base of most setae, hairs, and scales strongly expressing Gal4 (Figure 12, D–I). Gal4 expression is seen in nerve-rich regions of the leg, maxillary palps, and antennae (Figure 12, F–H). Cells of the base of every scale on the wings express Gal4 (Figure 12, I and J).

DISCUSSION

The functionality of the Gal4/UAS transcription regulatory system has been demonstrated in a range of eukaryotes, and when coupled to transposons, it becomes a powerful technology for the purposes of scanning genomes for the presence of gene regulatory elements and then using those regulatory elements to control transgene expression. Although useful, Gal4-based enhancer-trapping systems have been developed for few insects other than D. melanogaster, yet such systems powerfully complement existing efforts to manipulate insect genomes and determine the function of insect genes (Brand and Perrimon 1993; Trauner et al. 2009; Uchino et al. 2008). That such a system is now available for a major vector of human pathogens is of some significance given the interest in manipulating the genome of Anopheles mosquitoes not only for the purposes of advancing the functional genomics analysis of these insects but also for the development of novel strategies for controlling vector populations and their capacity to transmit parasites such as Plasmodium (Catteruccia 2007).

The results presented here show that when coupled to piggyBac transposons and introduced into the genome of An. stephensi, the Gal4/UAS system can be used to readily detected enhancers with a wide variety of activities. In this system, the piggyBac transposase promoter located in the 5’ subterminal region of the element was used to provide essential basal promoter functions for Gal4 gene regulation. This configuration of the Gal4 enhancer-detection system in An. stephensi is similar to the P-element–based enhancer-trap system widely used in D. melanogaster, which utilizes the P-element transposable promoter to provide essential basal promoter functions for Gal4 gene expression (Brand and Perrimon 1993). In both systems, the transposase promoters are weakly active and do not result in detectable levels of Gal4 expression in larval or adult tissues in the absence of enhancers.

The frequency of enhancer detection in An. stephensi using the system described here was high enough to allow for the rapid generation and detection of enhancer-trap events. Of the approximately 24,000 progeny screened in this study as both larvae and adults that could potentially harbor an enhancer-trap event,
approximately 300 were found with novel expression patterns of the reporter gene \textit{tdTomato} due to the influence of an enhancer (317/24,250; 1.3%). As we reported in an earlier study (O’Brochta \textit{et al.} 2011), we observed more \textit{piggyBac} remobilization events when the system was in the germ line of females compared with males (Table 2). Although we observed these differences consistently and the differences were statistically significant, the biological basis and significance of these observations remain unknown, and additional data are needed to address this question. Because all matings in this study were performed \textit{en masse}, we were unable to estimate the frequency of germ lines yielding enhancer-trap events; therefore, direct comparisons of the performance of this enhancer-trap system with the systems described for \textit{Drosophila}, \textit{Tribeilium}, and \textit{Bombyx} cannot be made (Trauner \textit{et al.} 2009; Uchino \textit{et al.} 2008). \textit{Anopheles stephensi} is highly fecund in the laboratory, with females producing some 350 progeny over three gonotrophic cycles following blood meals, which means that genetic screens involving tens of thousands of progeny are practical. The number of progeny arising from each enhancer-trap event within a genome (sometimes referred to as “cluster size”) was very small in the genetic screens reported here. Multiple progeny with an identical pattern of \textit{tdTomato} expression, containing \textit{piggyBac} in the same genomic position and found among the progeny of a single genetic cross, were rarely recovered. At this point, the temporal patterns of \textit{piggyBac} transposition within the germ line of \textit{An. stephensi} are unknown, although our observations suggest that transpositions are not occurring early during germ line development. The promoter from the \textit{hsp70} gene from \textit{D. melanogaster} regulates the \textit{piggyBac}
transposase transgenes in lines F2 and M5; however, its expression did not require heat induction in *An. stephensi*. Future studies will explore the relationship between the timing and frequency of heat shock and the amount and timing of piggyBac remobilization and enhancer detection. Despite the fact that there are aspects of this enhancer-trap system that remain to be determined, it promises to be quite useful for creating Gal4-expressing *An. stephensi* lines with widespread utility.

The genetic manipulation of *An. stephensi* by vector biologists remains somewhat challenging because there are relatively few promoters that have been isolated and characterized, and the creation of transgenic *An. stephensi* remains technically demanding. Most transgenic lines of *An. stephensi* created to date have been single-purpose lines with limited utility beyond their intended function, which further increased the costs and inefficiencies associated with using transgenic technologies in this species. The development and use of a Gal4-based enhancer-trap system increases the utility of transgenic technologies in *An. stephensi* by providing researchers with many more options for expressing transgenes of interest in temporal and spatial patterns. For example, we described lines in which enhancers were detected that regulated Gal4 expression in the adult female midgut, salivary gland, and fat body, three tissues that play critical roles in Plasmodium infection and transmission. Lines UMITF-C2F8 and UMITF-2MC14 had Gal4 expression in the midgut but not in the salivary glands or fat body of adult females. Lines UMITF-C2F41 and UMITF-2MC6 had Gal4 expression in the salivary glands but not in the midgut or fat body of adult females. Line UMITF-AEA1 had Gal4 expression in the fat body but no expression in the midgut and only weak expression in the salivary glands of adult females. Line UMITF-MBL24 was particularly interesting from the perspective of Plasmodium infection of *An. stephensi* because Gal4 expression occurred specifically in the adult female salivary glands, posterior midgut, and fat body. This line will permit transgenes to be expressed in three of the most important tissue compartments of *An. stephensi* with respect to Plasmodium infection within a single adult female. Line UMITF-MDL8 is expected to be useful because it has Gal4 expression ubiquitously throughout most tissues of both larvae and adults. Although most of the lines reported here were chosen to illustrate the utility of this technology to the study of mosquito-parasite/pathogen interactions, lines UMITF-C2M8 and UMITF-DEA9A had Gal4 expression exclusively or almost exclusively in larval tissues, whereas line UMITF-2MBL3 had Gal4 expression in a specific subset of cells associated with scales and sensillae. The binary nature of this system permits the effort spent on creating transgenic lines to be minimized while enabling investigators repeated opportunities to express their transgene in a variety of patterns simply by mating their Gal4-regulated transgene–containing line to any Gal4-expressing line. This modularity is perhaps the most important feature of this system.

The system described here, although effective at detecting enhancers, could be made more effective with two modifications. First, piggyBac transposase is currently not limited to the germ line of lines M5 and F2. Because the transposition activity of the enhancer-trap element is sufficiently high in somatic cells, clones of Gal4-expressing cells in various tissues are frequently seen (Figure 2). If enhancer-trap events, in which the expected Gal4 expression patterns involved relatively small numbers of cells resulting in a subtle but significant pattern of reporter gene expression, are of interest, then the somatic clones frequently observed with our current system could be a liability by making such patterns difficult to recognize. Limiting transposase expression to the germ line of *An. stephensi* could be accomplished by using regulatory sequences that result in germ line–specific transcription (Papathanos et al. 2009). Our current enhancer-trap system has also shown that having the UAStdTomato transgene in a piggyBac vector can be disadvantageous. For example, when piggyBac transposase is present, the UAS-containing piggyBac element can become unstable and be remobilized to new genomic locations. Also, performing splinkerette-PCR or using any other method to identify integration sites of piggyBac elements containing the enhancer-reporter can be confounded by the presence of piggyBac elements containing UAS-regulated reporter genes. Although this current system could be improved by incorporating all UAS-regulated reporter genes into vectors other than piggyBac, careful genetics and accounting for chromosomes containing the piggyBac transposase transgene will avoid any undesirable remobilization of other system components. Although having other transposon platforms upon which to build system components is convenient, the highly effective enhancer-trap systems created for
Anopheles mosquitoes using forward and reverse genetic approaches. This is valuable for empirically assessing gene function within the context of genomics. For Anopheles mosquitoes, there are relatively few tools available for empirically assessing gene function within the context of the whole organism. The enhancer-trap system described here is a valuable first step in increasing our capacity to explore the biology of Anopheles mosquitoes using forward and reverse genetic approaches.

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**Figure 12** Line UMITF-2MBL3. (A) Dorsal view of a fourth instar larva. (B) Higher magnification of the dorsal side of the head of the larva in (A). No tdTomato expression is observed in the brain. (C) Higher magnification of the ventral side of the head of the larva in (B), tdTomato expression is seen in the setae associated with the mandibles (man) and at the base of setae (s) in the thorax. (D) Dorsal view of an adult female. (E) Ventral view of an adult female. (F) Tarsus from an adult female. (G) Maxillary palp from an adult female. (H) Antenna from an adult female. (I) Dorsal view of a wing from an adult female. (J) Same as (I) but under UV light to show tdTomato-expressing cells.

D. melanogaster were based on a single transposon platform (Bellen 1999).

The abundance of genome information and the ease with which it can now be obtained makes the need eminent for technologies that enable progress to be made in pursuing questions relating to functional genomics. For Anopheles mosquitoes, there are relatively few tools available for empirically assessing gene function within the context of the whole organism. The enhancer-trap system described here is a valuable first step in increasing our capacity to explore the biology of Anopheles mosquitoes using forward and reverse genetic approaches.
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