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Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system

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Abstract

In this study, we report the establishment of the binary Gal4/UAS system for the yellow fever mosquito *Aedes aegypti*. We utilized the 1.8-kb 5' upstream region of the vitellogenin gene (*Vg*) to genetically engineer mosquito lines with the *Vg-Gal4* activator and established *UAS-EGFP* responder transgenic mosquito lines to evaluate the binary Gal4/UAS system. The results show that the *Vg-Gal4* driver leads to a high level of tissue-, stage- and sex-specific expression of the *EGFP* reporter in the fat body of *Vg-Gal4/UAS-EGFP* hybrids after blood-meal activation. In addition, the applicability of this system to study hormonal regulation of gene expression was demonstrated in *in vitro* organ culture experiments in which the *EGFP* reporter was highly activated in isolated fat bodies of previtellogenic *Vg-Gal4/UAS-EGFP* females incubated in the presence of 20-hydroxyecdysone (20E). Hence, this study has opened the door for further refinement of genetic tools in mosquitoes.

Keywords

genetic transformation; vitellogenin promoter; piggyBac transposable element

1. Introduction

Mosquitoes transmit numerous pathogens of devastating human diseases. Advances in mosquito molecular biology, genetics and genomics have been stirred by demands to develop novel and perfect already-existing approaches for mosquito control. Genomes have been sequenced for three mosquitoes: *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*, vectoring malaria, Dengue and Yellow fevers, and West Nile virus, respectively (Arensburger et al., 2010; Holt et al., 2002; Nene et al., 2007). Introduction of RNA interference reverse genetics (RNAi), microarray and RNA-sequence technologies has empowered vector research (Blandin et al., 2002; Dimopoulos et al., 2002, Bonizonni et al., 2011; Niera-Ovieda et al., 2011), and development of genetic transformation in mosquitoes has played a significant role in enriching a genetic toolbox available for mosquito research (Catteruccia et al., 2000; Coates et al., 1998; Jasinskiene et al., 1998; Kokoza et al., 2000; Kokoza et al., 2001a; Kim et al., 2004). During the last decade, great progress has been achieved in the development of these novel strategies for potential mosquito and pathogen

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control (James, 2005; Terenius et al., 2008). A transgenic approach was used in studies of mosquito immunity (Antonova et al., 2009; Bian et al., 2005; Shin et al., 2003; Shin et al. 2006; Cheon et al., 2006), and transgenesis has been instrumental in demonstrating a proof-of-principal concept of creating a vector with elevated resistance to a malaria pathogen (Ito et al., 2002; Kim et al., 2004). Using RNA interference techniques, there has been success in engineering partial Dengue virus resistance in *Ae. aegypti* (Franz et al., 2006; Mathur et al., 2010). Kokoza et al. (2010) showed that co-overexpression of two effector molecules with anti-pathogen action in the same transgenic mosquito results in a total refractory phenotype. Transgenesis has recently been used to engineer a repressive, female-specific, flightless phenotype in *Ae. aegypti* mosquitoes; this holds promise for control of mosquito populations (Fu et al., 2010). Transgenic manipulation of regulatory pathways opens the possibility of decreasing the life span of mosquitoes (Corby-Harris et al., 2010).

Despite this impressive gain in knowledge of molecular genetics of mosquitoes, there is an urgent need to develop and refine methods of genetic engineering in these disease vectors. It is of particular importance for evaluating genes that have detrimental effects on development, reproduction, nutritional and metabolic processes, and other aspects of mosquito biology.

One powerful genetic approach is the conditional targeting of gene expression using the binary Gal4/UAS system (Ornitz et al., 1991, Brand and Perrimon, 1993). This system has been essential for genetic research in *Drosophila* (Brand and Perrimon, 1993, Duffy, 2002; McGuire et al., 2004), as well as several other model organisms, such as mice (Ornitz et al., 1991; Mallo, 2006), zebra fish (Scheer and Campos-Ortega, 1999; Baier and Scott, 2009), *Xenopus* (Hartley et al., 2002) and *Arabidopsis* (Guyer et al., 1998). Among non-Drosophilid insects, establishment of the binary Gal4/UAS system has been reported for the silkworm, *Bombyx mori* (Imamura et al., 2003) and, recently, for the beetle *Tribolium castaneum* (Schinko et al., 2010). The established binary Gal4/UAS system is based on the production of two independent transgenic lines: one with the yeast *Gal4* activator gene under a tissue-, cell-specific, inducible or constitutive promoter (the driver line), and another with the binding sites for Gal4 protein—the *upstream activation sequence* (*UAS*), which can control the expression of any gene of interest (the responder line). Crossing these two lines results in expression of a reporter gene in a hybrid organism in a manner that depends on a promoter used in the driver line (Brand and Perrimon, 1993). The binary Gal4/UAS has been essential for deciphering numerous gene functions (Duffy, 2002; McGuire et al., 2004; Mallo, 2006; Baier and Scott, 2009). The major advantage of this system is that, without an activator, no expression of the gene of interest takes place. This allows overexpression of genes with potentially harmful effects on development, behavior and fertility. Importantly, with the Gal4 binary system, it is possible to study a phenotypic effect of dominant lethal genes, producing conditional lethality in specific tissue and at a specific stage. Introduction of this technology along with characterization and cloning of an ever-increasing list of genes with specific stage, sex, tissue or cell patterns of expression has revolutionized molecular genetics of model organisms.

In this study, we report the establishment of the binary Gal4/UAS system for the yellow fever mosquito *Ae. aegypti*. This represents a significant step in further refinement of genetic tools in mosquitoes.

2. Experimental Procedures

2.1. Mosquito rearing

The *Ae. aegypti* wild-type UGAL/Rockefeller strain and transgenic lines (the driver Vg-Gal4 and the responder UAS-EGFP) were reared at 27°C and 80% humidity, as described

previously (Roy et al., 2007). Blood feeding of adult mosquitoes (4- to 5-day-old females) was performed using White Leghorn chickens.

2.2. DNA constructs

The germ-line transformation vectors carrying the *Vg-Gal4* driver and *UAS-EGFP* responder (Fig.1) were constructed as described below. The Vg-Gal4 driver cassette was assembled in the shuttle vector pSLfa (Horn and Wimmer, 2000). The 1.8-kb *BglII-Bam HI* fragment of the *Vg* 5' promoter region (Kokoza et al., 2001b) was linked with a 0.8-kb *BamHI-XbaI* fragment of the chimeric Gal4 activator excised from the modified pTwiggy, one of the *Drosophila* transformation vectors with a driver construct (Arnosti et al., 1996, Kulkarni and Arnosti, 2003). In this version of the Gal4 activator, two essential domains, the DNA-binding (amino acids, 1–93) and activation (amino acids, 753–881), were fused directly, resulting in the chimeric Gal4Δ activator widely used in *Drosophila* studies as a more effective driver than the original Gal4 protein (Kulkarni and Arnosti, 2003). The resulting *Vg-Gal4* driver construct was subcloned from the pSLfa shuttle plasmid into the pBac [3xP3-EGFP *afm*] transformation vector in the *Asc*I unique cloning site. The pBac [3xP3-EGFP *afm*, *Vg-Gal4*] construct has a marker EGFP gene driven by the *3xP3* eye-specific promoter and was used for germ-line transformation to produce the driver transgenic line.

The UAS-EGFP responder cassette was made by linking the 0.5-kb *Bam*HI-*Bgl*II fragment of pUAST vector (Brand and Perrimon, 1993) with a 0.75-kb EGFP sequence, followed by a 0.25-kb SV40 polyadenylation signal in the pSLfa shuttle plasmid. The 5' region of this construct has five tandemly arrayed GAL4 binding sites, the *upstream activation sequence* (*UAS*), followed by *Hsp70* basal promoter with TATA box and a 68-bp leader sequence (Brand and Perrimon, 1993). The activation of this 5' upstream region by the DNA binding domain of the GAL4 protein drives the *EGFP* gene expression as a reporter part of the construct. The 1.5-kb UAS-EGFP responder cassette was introduced into the pBac[3xP3-DsRed *afm*] transformation vector (Horn and Wimmer, 2000) at the unique *Asc*I site. The resulting pBac[3xP3-DsRed a*f*m, *UAS-EGFP*] plasmid has a marker DsRed gene driven by a 3xP3 eye-specific promoter and was used for mosquito germ-line transformation to produce a responder line.

2.3.Germ-line transformation

The germ-line transformation was performed as described previously (Kokoza et al., 2001). Briefly, the DNA of the pBac [3xP3-EGFP *afm*, *Vg-Gal4*] driver, pBac[3xP3-DsRed a*f*m, *UAS-EGFP*] responder, and phsp-pBac helper plasmid (Handler and Harrell, 1999) was purified using a QIAFilter kit (QIAGEN) and dissolved in 0.1mM phosphate buffer (pH 6.8), 5mM KCl. The mixture of the vector and helper DNA, at the final concentrations of 0.35mg/ml and 0.25mg/ml, respectively, was injected into pre-blastoderm eggs of the wild-type UGAL/Rockefeller strain. Eggs for injection experiments were collected 72 h post-blood meal (PBM). The development of Vg-Gal4 driver and UAS-EGFP responder transgenic lines was done essentially as described previously (Kokoza et. al., 2001a), based on G_1 progeny selection, using the EGFP and DsRed 3xP3 eye-specific selectable markers (Horn et al., 2002). The hybrid line *Vg-Gal4/UAS-EGFP* hybrid mosquitoes was established as previously described (Kokoza et al., 2010).

2.4. Molecular analysis (Genomic PCR, Inverse PCR and RT-PCR)

Genomic DNA from transgenic and wild-type mosquitoes was purified using the DNeasy tissue kit (QIAGEN). PCR was performed using 50 ng of genomic DNA isolated from adult mosquitoes as a template. The primers used for gene amplification are listed in Table S1.

PCR was done as follows: denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 40 s and 68 °C for 1 min, using Platinum *Taq* polymerase (Invitrogen).

Inverse PCR analysis was done using genomic DNA (5 ng) from transgenic Vg-Gal4 and UAS-EGFP mosquitoes using the *Taq*I and *Msp*I endonuclease digestion to identify 5' and 3' junctions, respectively. The inverse PCR reaction was performed essentially as previously described (Handler et. al., 1998; Kokoza et al., 2001), with primers specific to the left and right arm of the *piggyBac* transposon (Table S1). The PCR products were cloned in a TA vector (Invitrogen) and the sequence from plasmid DNA of the five to seven independent clones was determined for each junction.

Total RNA from the transgenic and wild-type mosquitoes was isolated by the Trizol reagent (Invitrogen) using the manufacturer's protocol. Contaminating genomic DNA was removed from the RNA preparation by treatment with DNAse I (GIBCO BRL/Invitrogen). The cDNA was synthesized using the Omniscript Reverse transcriptase kit (Qiagen), in a 20-µl reaction mixture containing 1 µg of the total RNA and oligo-dT primers, via incubation at 37 °C for 1 h. The PCR reactions were performed as previously described (Kokoza et. al., 2000, 2001a) using gene-specific primers (Table S2). To detect the transgenic transcripts, we designed a pair of primers that specifically recognized Vg-Gal4 or UAS-EGFP mRNA. For driver construct, the 5' leader sequence of Vg mRNA was used as forward primer, and the sequence from the Gal4 protein reading frame was used as reverse. To detect the expression of the responder construct, the forward primer was designed from the 5' leader sequence of hsp70 mRNA present in the *UAS* fragment, and the reverse primer was from the EGFP protein-coding sequence.

2.5. Microscopy and Epifluorescence Analysis

The expression of EGFP from the driver construct pBac[3xP3-EGFP *afm*, *Vg-Gal4*] and expression of DsRed from the responder pBac[3xP3-DsRed a*f*m, *UAS-EGFP*] in the transgenic mosquitoes were detected using a Nikon SMZ800 fluorescence microscope equipped with a GFP-B and DsRed filter set, respectively. The images were captured using a Nikon DXM 200 digital system. This system was also used for visual observation of the adult females to detect EGFP expression in the fat body of transgenic and wild-type mosquitoes after blood meal activation.

For EGFP fluorescence detection in the previtellogenic fat body of Vg-Gal/UAS-EGFP hybrids, after *in vitro* incubation with the hormone 20E, the individual fat bodies were first fixed in 3.7% formaldehyde in Aedes physiological saline (APS) buffer solution for 20 min at room temperature. They were then washed three times for 10 min in APS and transferred into a DNA-staining solution containing 5µM Hoescht 33342 (H1399, Invitrogen) in APS for 10 min. Finally, the fat bodies were mounted in VectaShield-H1000 (Vector Laboratories) mounting media for the fluorescence, and imaging was performed under a Zeiss AxioObserver A1 microscope, using AxioVision software.

2.6. In Vitro Fat Body Culture

In vitro fat body culture was performed as previously described (Raikhel et.al., 1997; Roy et al., 2007). An established system contained a mixture of all amino acids (120 nM/µl) in the incubation medium in the presence or absence of 20E (Sigma) with a final concentration of 10 µM. Fat bodies isolated from previtellogenic females 3–5 days post-eclosion were incubated in the *in vitro* culture medium in the presence or absence of 20E for 6–8 h at 27 °C in an incubator. After incubation, the fat bodies were harvested and used to isolate RNA by means of the Trizol method, or prepared for fluorescence microscopy analysis.

3. Results

3.1. Transformation experiments and genomic analysis

To generate the transgenic *Ae. aegypti* Gal4 driver line, we first constructed a transposon based on a *piggyBac* transposable element (Fig. 1A). This *Vg-Gal4* driver construct included a mosquito 1.8-kb *Vg* gene promoter region linked to the modified yeast *Gal4* protein coding sequence containing the DNA binding domain (amino acid residues 1–93) and the activation domain (amino acid residues 753–881), followed by the SV40 polyadenylation signal. The driver construct also carried a selectable *EGFP* marker gene under the 3xP3 eye-specific promoter.

The *piggyBac*-based responder construct contained 5xUAS—five repeat concatamers of the consensus binding site for Gal4 with TATA box and the *hsp70* minimal promoter linked to the *EGFP* reporter gene and SV40 polyadenylation sequence. This responder transposon included a selectable *DsRed* marker gene under the 3xP3 eye-specific promoter (Fig. 1B).Germ-line transformation experiments were performed using a technique developed previously (Kokoza et al., 2001a).

Of 800 embryos injected with the *Vg-Gal4* driver construct, 129 survived to adulthood and gave progeny (Table S3). The G_0 mosquitoes were outcrossed with the host wild-type UGAL/Rockefeller strain, and G_1 progeny (larvae and pupae) were examined for EGFP in their eyes (Fig. S1A). Of 1300 embryos injected with the *UAS-EGFP* responder construct, 240 mosquitoes became adult. Transformants for the *UAS-EGFP* responder were selected for the presence of DsRed marker in their eyes (Fig. S1B). Two independent *Vg-Gal4* driver and four *UAS-EGFP* responder transgenic lines were initially selected for further analyses (Table S2). Transformation efficiency was approximately 1.5% in both experiments.

For further analyses, we selected two lines based on a strong expression of eye-specific marker genes; one for the *Vg-Gal4* driver (DA55) and another for the *UAS-EGFP* responder (R-234). Both these lines were established as homozygous for respective transgenes. We considered these lines to be homozygous based on several criteria. After four generations of selection, 100% of mosquitoes in each line exhibited the eye-specific markers. In outcross experiments, individual transgenic males were crossed with wild-type females; and transgenic females to wild-type males in reciprocal crosses. All progeny, originated from individual parents, had eye-specific markers; EGFP for the *Vg-GAL4* driver and DsRed for the *UAS-EGFP* responder. Our molecular analysis using inverse PCR suggested that each line had a single copy insertion of the piggyBac transposon (Table S3). For both the DA55 driver line and the R-234 responder line, all sequenced plasmids had the same target sites of their respective transgenes confirming the unique single copy incorporation. Incorporation of respective transgenes into both DA55 driver and R-234 responder lines occurred via a *bona fide*, canonical, duplicated TTAA target site characteristic of the *piggyBac*-mediated insertion (Table S3).

Genomic PCR analysis was conducted to confirm a stable incorporation and the integrity of *Vg-Gal4* and *UAS-EGFP* constructs into the *Ae. aegypti* genome in the DA-55 driver and the R-234 responder lines. For each line, the genomic DNA was isolated from 7–10 transgenic mosquitoes, and aliquots of these samples were analyzed by PCR using a pair of primers specific for *piggyBac* transposon left arm, right arm, *Vg-Gal4* driver and *UAS-EGFP* responder sequences (Fig. 2). The integrity of isolated genomic DNA was confirmed by PCR amplification of the endogenous actin gene as a positive control. The DNA isolated from wild-type (UGAL) mosquitoes was used as a negative control (Fig. 2). The transgene-specific primer sets confirmed the presence of *piggyBac* transposon sequences (left arm and right arm) in genomic DNA isolated from mosquitoes of DA55 and R-234 lines (Fig. 2A

and 2B). *Vg-Gal4* driver sequence was present in genomic DNA isolated from of the DA55 line, while the *UAS-EGFP* responder sequence was present in DNA from the R-234 line. No amplification was observed in genomic DNA isolated from the non-transgenic parental UGAL strain (Fig. 2D).

After stable *Vg-Gal4* driver and *UAS-EGFP* responder homozygous lines had been established, we produced *Vg-Gal4/UAS-EGFP* hybrid mosquitoes by crossing these two transgenic lines and selecting mosquitoes with the simultaneous presence of both EGFP and DsRed eye-specific markers (Figs. S1C, D). Genomic PCR analysis confirmed the presence of *piggyBac* transposon sequences (left arm and right arm) in genomic DNA isolated from *Vg-Gal4/UAS-EGFP* hybrid mosquitoes. Moreover, both *Vg-Gal4* and *UAS-EGFP* sequences were present in genomic DNA from the hybrid mosquitoes (Fig. 2). There were no detectable negative effects on mosquito development, egg production, viability or sex ratio in either of these two transgenic lines, or in the *Vg-Gal4/UAS-EGFP* hybrid mosquitoes (data not shown).

3.2. Expression Analysis

To demonstrate whether the constructed Gal4/UAS system worked in *Ae. aegypti* mosquitoes, we carried out a detailed expression analysis. The RT-PCR analysis was performed using a pair of primers that recognized the transgenic *Vg-Gal4* hybrid transcripts specifically and not the endogenous *Vg* mRNA (Kokoza et al., 2000; 2001a). The parental UGAL strain was used as a negative control of expression in all experiments. No *Vg-Gal4* transcript was observed at the previtellogenic stage of any transgenic line. The *Vg-Gal4* transcript was found in the fat body of the transgenic female mosquitoes of the DA-55 driver line after a blood meal, when it reached a peak at 24 h PBM in accordance with the expression of the *Vg* promoter driver. The *Vg-Gal4* transcript had a similar pattern in the *Vg-Gal4*/*UAS-EGFP* hybrids (Figs. 3A, B). No expression of the transgenic *Vg-Gal4* activator transcript was observed in the responder *UAS-EGFP* line or the parental UGAL mosquitoes (Figs. 3C, D). These results clearly demonstrate that the transgenic *Vg-Gal4* activator is transcribed PBM in both the DA55 driver line and the *Vg-Gal4*/*UAS-EGFP* hybrid mosquitoes.

The RT-PCR analysis using EGFP-specific primers showed that this transcript was found only in the hybrid *Vg-Gal4/UAS-EGFP* transgenic mosquitoes (Fig. 3A). EGFP mRNA expression was detected in neither the DA-55 driver nor the R-234 responder lines (Figs. 3B and C). No EGFP mRNA was seen in the UGAL parental mosquitoes (Fig. 3D). The EGFP transcript was found only in the fat body, not in the ovary, midgut, or Malpighian tubules of the *Vg-Gal4*/*UAS-EGFP* female mosquitoes (Fig. 4). Males of the same hybrid line were negative for the EGFP transcript (Fig. 4). These data indicate that transactivation of the reporter gene in the established Gal4/UAS binary system occur in a tissue-, sex-, and stage-specific manner and resemble the expression of the endogenous *Vg* gene (Figs. 3 and 4).

Microscopic fluorescence analysis was used to establish that the reporter mRNA was not only transcribed but also was efficiently translated in the *Vg-Gal4*/*UAS-EGFP* female mosquitoes. Strong EGFP fluorescence was observed in abdomens of these mosquitoes after blood feeding; it reached a maximum at 24 h PBM and persisted until 48 h PBM (Fig. 5). No positive signal was observed in females of the driver DA-55 driver, the R-234 responder lines or UGAL mosquitoes (Fig. 5).

3.3. In vitro activation of the Vg-Gal4/UAS expression system in the fat body by 20E

20E is the major regulator of mosquito vitellogenesis (Raikhel et al., 2005). We conducted *in vitro* tissue culture experiments to determine whether 20E activates *Vg-Gal4/UAS-EGFP*

in the fat bodies of transgenic mosquitoes (Raikhel et al., 1997; Roy et al., 2007). Fat bodies isolated from previtellogenic *Vg-Gal4/UAS-EGFP* hybrid female mosquitoes were incubated for 6 h in culture medium in the presence or absence of 20E. We performed RT-PCR analysis utilizing gene-specific primers to *UAS-EGFP* reporter, *Vg-Gal4* and *actin* as a loading control. This analysis revealed the presence of high levels of both *UAS-EGFP* and *Vg-Gal4* transcripts in fat bodies from previtellogenic *Vg-Gal4/UAS-EGFP* female mosquitoes treated with 20E (Fig. 6). Thus, the *Vg-Gal4/UAS-EGFP* system was activated by the hormone treatment at the transcriptional level.

To determine whether 20E-activated expression of the *Vg-Gal4/UAS-EGFP* resulted in translation of EGFP, we conducted fluorescence microscopy analysis of the fat bodies isolated from the previtellogenic *Vg-Gal4/UAS-EGFP* females after *in vitro* hormonal treatment with 20E, as described above. This visual analysis revealed a high level of GFP fluorescence in the fat body tissue (Figs. 7A, B, C; Fig. 8A). No EGFP fluorescence was observed in fat bodies from the DA-55 driver, R-234 responder or parental UGAL mosquitoes (Figs. 8B, C, D).

4. Discussion

We have reported here the establishment of the binary Gal4/UAS system for the yellow fever mosquito *Ae. aegypti*. The results show that the *Vg-Gal4* driver leads to a high level of tissue-, stage- and sex-specific expression of the *EGFP* reporter in the fat body of *Vg-Gal4/UAS-EGFP* mosquitoes after blood-meal activation. We utilized the 1.8-kb 5' upstream regulatory region of the *Vg* gene, which has been shown be sufficient to lead expression of a transgene in manner similar to that of the endogenous *Vg* gene (Kokoza et al., 2000; 2001a; 2001b). Activation of the *Vg* gene was shown to depend on ingestion of blood by female mosquitoes, and it was expressed in a strict sex-specific, tissue-specific and temporal manner. The level of its expression increased dramatically by 24 h PBM and then declined to a background level by 36–42 h PBM. The *Vg* gene was expressed exclusively in the female fat body, which is the tissue central to reproduction, immunity and metabolism. The *Vg* promoter provides an ideal driver for expression of genes of interest in this important tissue, as it has been demonstrated in previous studies (Antonova et al., 2009; Bian et al., 2005; Shin et al., 2003; Shin et al. 2006; Cheon et al., 2006; Kokoza et al., 2010). The *EGFP* transcript was present only in fat bodies of female mosquitoes after a blood meal, with its maximal level at 24 h PBM. Moreover, the EGFP protein was translated, as judged by EGFP fluorescence in the abdomens of *Vg-Gal4/UAS-EGFP*. Due to stability of EGFP, the fluorescent signal was observed in female mosquitoes even at 48 h PBM. A similar prolonged presence of an expressed product had been reported previously for Defensin in the transgenic *Ae. aegypti* (Kokoza et al., 2000). Thus, our *Vg-Gal4/UAS-EGFP* system is ready for expression of target genes, their modifications, or target gene depletions. The development of the mosquito GaL4/UAS system will clearly lead to: 1) identification and *in vivo* testing of robust stage- and tissue-specific promoters; and 2) characterization of genes critical for reproduction or survival of a mosquito.

Initiation and maintenance of the *Vg* gene expression is accomplished by the cooperative action of the nutritional amino acid/Target of Rapamycin signaling and 20E pathways (Attardo et al., 2005; Raikhel et al., 2005). The 20E regulatory hierarchy controls *Vg* gene expression via transcription factors: the ecdysone receptor (EcR/USP), E74 and Broad (Raikhel et al., 2002; 2005). *cis* binding sites complementary to these transcription factors are present in the 1.8-kb *Vg* 5' upstream region used for creating the *Vg-GAL4* driver. *In vitro* organ culture experiments utilizing fat bodies from previtellogenic *Vg-Gal4/UAS-EGFP* females have shown that *Vg-Gal4* is responsive to the physiological dose of 20E in the presence of amino acids and activated the *EGFP* gene via GAL4–UAS interaction.

EGFP protein was translated, resulting in an intense fluorescence of the incubated fat bodies. Hence, our data expand the applicability of the established *Vg-Gal4/UAS* system not only for an *in vivo* analysis, but also for *in vitro* tissue culture studies of gene expression regulation.

Transformation efficiencies via transposable elements for non-Drosophilid insects are low. In this work, it was 1.5% in both transformation vectors. This is in a range of efficiency typical for mosquito species (Kokoza et al., 2001a, Kim et. al., 2004; Lobo et al., 2002; Labbe et al., 2010). Similar transformation efficiencies have been observed in other insects, such as 2.5–2.7% in *Bombyx mori* (Imamura et al., 2003), 3–5% in the Mediterranean fruit fly (Handler et al., 1998) and 4–5% in *Tribolium castaneum* (Victorinova et al., 2007). Recently, a tenfold increase in transformation efficiency has been reported using a newly developed hyperactive version of the piggyBac transposase (Yusa et al., 2010). Further advancements in genetic transformation tools are required to increase effectiveness of the GAL4/UAS system in non-Drosophilid insects.

Further expansion of research utilizing the binary Gal4/UAS system in mosquitoes to investigate their unique biology depends largely on characterization of tissue-, cell-, sex- and stage-specific genes, regulatory regions of which could be utilized as drivers for the binary mosquito Gal4/UAS system. A number of mosquito genes have been characterized and utilized in generating transgenic mosquitoes—*Ae. aegypti*, *An. stephensi* and *An. gambiae* (Kokoza et al., 2000, Ito et al., 2000, Cho et al., 2006; Adelman et al., 2007; Papathanos et al., 2009; Catteruccia et al., 2005; Smith et al., 2007; Nolan et al., 2011). However, further detailed studies are required to match the sophistication of genetic approaches utilizing the binary GAL4/UAS system in model organisms. Our study represents a significant step in the development of genetic tools in mosquitoes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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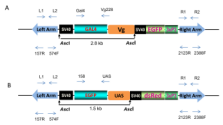

Fig. 1.

Schematic representation of two constructs, the pBac[3xP3-EGFP afm, *Vg-Gal4*] – driver (A) and the pBac[3xp3-DsRed af, *UAS-EGFP*] – responder (B) used in germ-line transformation experiments. The 2.8-kb driver transgene consists of the *Ae. aegypti* mosquito's *Vg*- 5' promoter region linked to yeast Gal4 activator sequence with DNA binding domain (amino acids, 1–93) and activation domain (amino acids, 753–881), followed by SV40 polyA signal (A). The 1.5-kb responder transgene contains the UAS sequence with 5× concatamers of the Gal4 binding sites fused to the EGFP reporter gene with SV40 polyA signal (B). The transgene-specific primers used for genomic PCR, RT-PCR and inverse PCR are indicated by the arrows above and below the diagram.

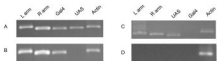

Fig. 2.

Stable incorporation and integrity of the *Vg-Gal4* driver and *UAS-EGFP* responder transposons into the *Ae. aegypti* genome. Genomic DNA was isolated from transgenic mosquitoes of the *Vg-Gal4/UAS-EGFP* hybrids (A), the driver line (B), the responder line (C), and non-transgenic UGAL strain (D). PCR amplification was performed using a set of primers specific to the left and right arms of the *piggyBac* vector (L arm and R arm), the *Vg-Gal4* transgene (Gal4) and the *UAS-EGFP* transgene (UAS). Primers to *Ae. aegypti* actin were used to confirm the integrity of genomic DNA.

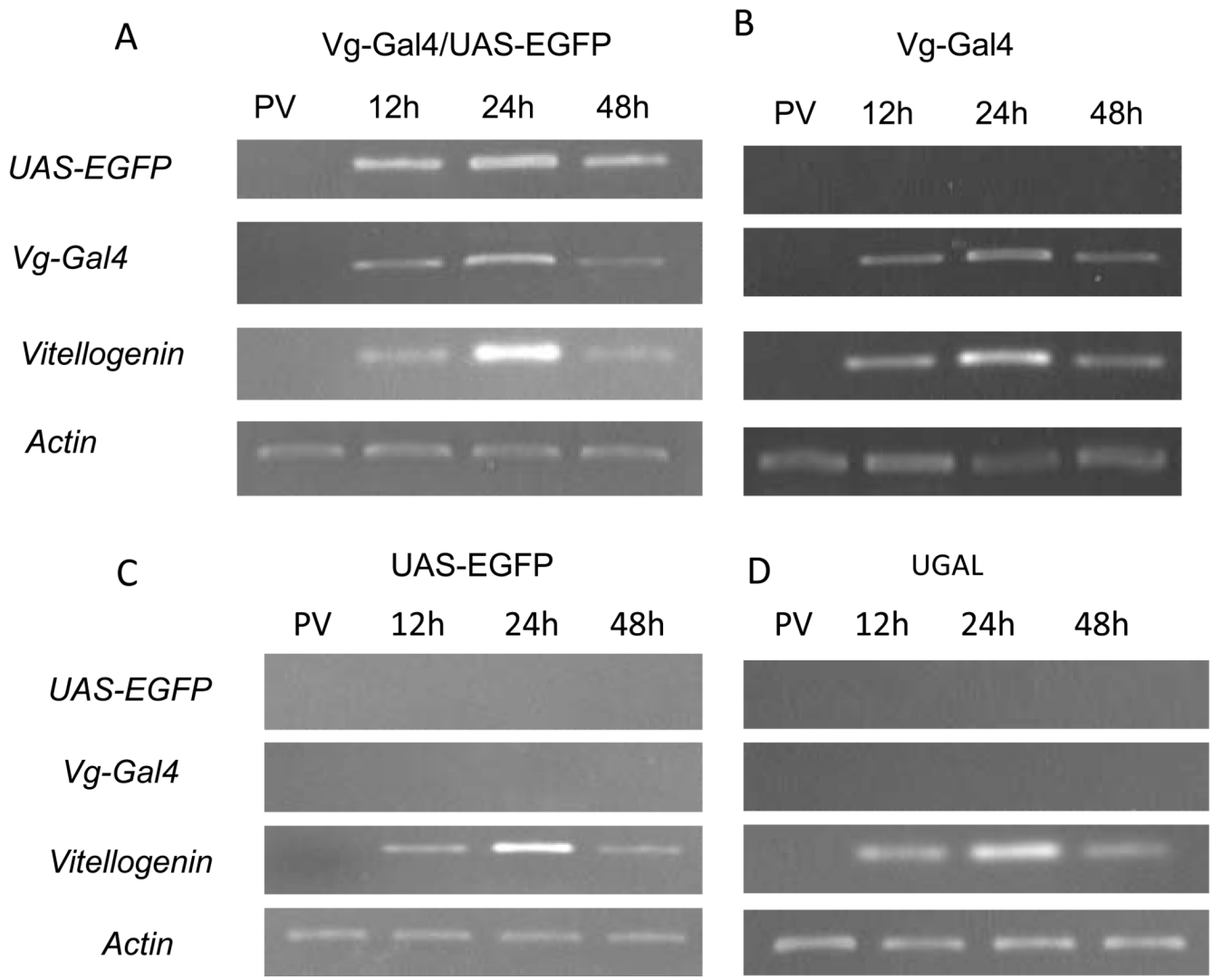

Fig. 3.

Blood meal activation of the binary *Vg-Gal4/UAS-EGFP* expression system in the fat body of the transgenic *Ae. aegypti* mosquitoes. RT-PCR analysis was performed using RNA samples isolated from the fat body of transgenic (A, B, C) and wild-type UGAL (D) mosquitoes. Expression profiles of the *Vg-Gal4*, *UAS-EGFP* transgenes, vitellogenin and actin in previtellogenic (PV) and vitellogenic females were analyzed 12, 24, and 48 h post-blood meal (PBM) using gene-specific primers.

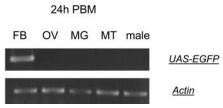

Fig. 4.

Tissue- and sex-specific expression of the reporter gene in the *Vg-Gal4/UAS-EGFP* hybrid mosquitoes. Fat bodies (FB), ovaries (OV), midgut (MG), and Malpighian tubules (MT) from vitellogenic *Vg-Gal4/UAS-EGFP* hybrid female mosquitoes, 24 h post-blood meal (PBM), were analyzed by means of RT-PCR analysis. Specific amplification of the EGFP reporter was detected only in vitellogenic fat bodies. Males (M) were also negative for EGFP reporter RNA. Actin-specific primers were used as a control for RNA integrity and loading.

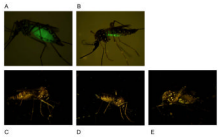

Fig. 5.

Tissue- and stage-specific EGFP reporter expression in the *Vg-Gal4/UAS-EGFP* hybrid female mosquitoes after blood meal activation. Fluorescent images of adult vitellogenic females were captured using an EGFP filter set. Expression of EGFP reporter was detected only in the fat body of hybrid *Vg-Gal4/UAS-EGFP* females 24 (A) and 48 (B) h post-blood meal (PBM). No EGFP fluorescence was observed in the fat body of vitellogenic (24 h PBM) females of the *Vg-Gal4* driver (C) and *UAS-EGFP* responder (D) transgenics, or non-transgenic UGAL strain (E).

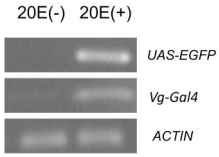

Fig. 6.

20E hormonal activation of reporter mRNA expression in the *Vg-Gal4/UAS-EGFP* hybrids in previtellogenic fat body in an *in vitro* organ culture. Previtellogenic fat bodies were incubated in culture media in the presence (20E+) or absence of (20E−) of 20E. RT-PCR analysis was performed using gene-specific primers to *UAS-EGFP* reporter, *Vg-Gal4* activator and actin as a loading control.

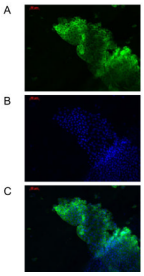

Fig. 7.

Fluorescent images of previtellogenic fat body of *Vg-Gal4/UAS-EGFP* hybrids after 20E hormonal activation in an *in vitro* organ culture. Fat bodies dissected from previtellogenic hybrid transgenic female mosquitoes were incubated in the culture medium in the presence of 20E and prepared for examination by fluorescence microscopy. The tissue was stained by Hoescht DNA staining for visualization of nuclei. Imaging was performed under a Zeiss AxioObserver A1 microscope using EGFP filter (A), Blue filter for nuclei staining (B), and both filters to obtain a merged image (C). All images have a 50-μm scale.

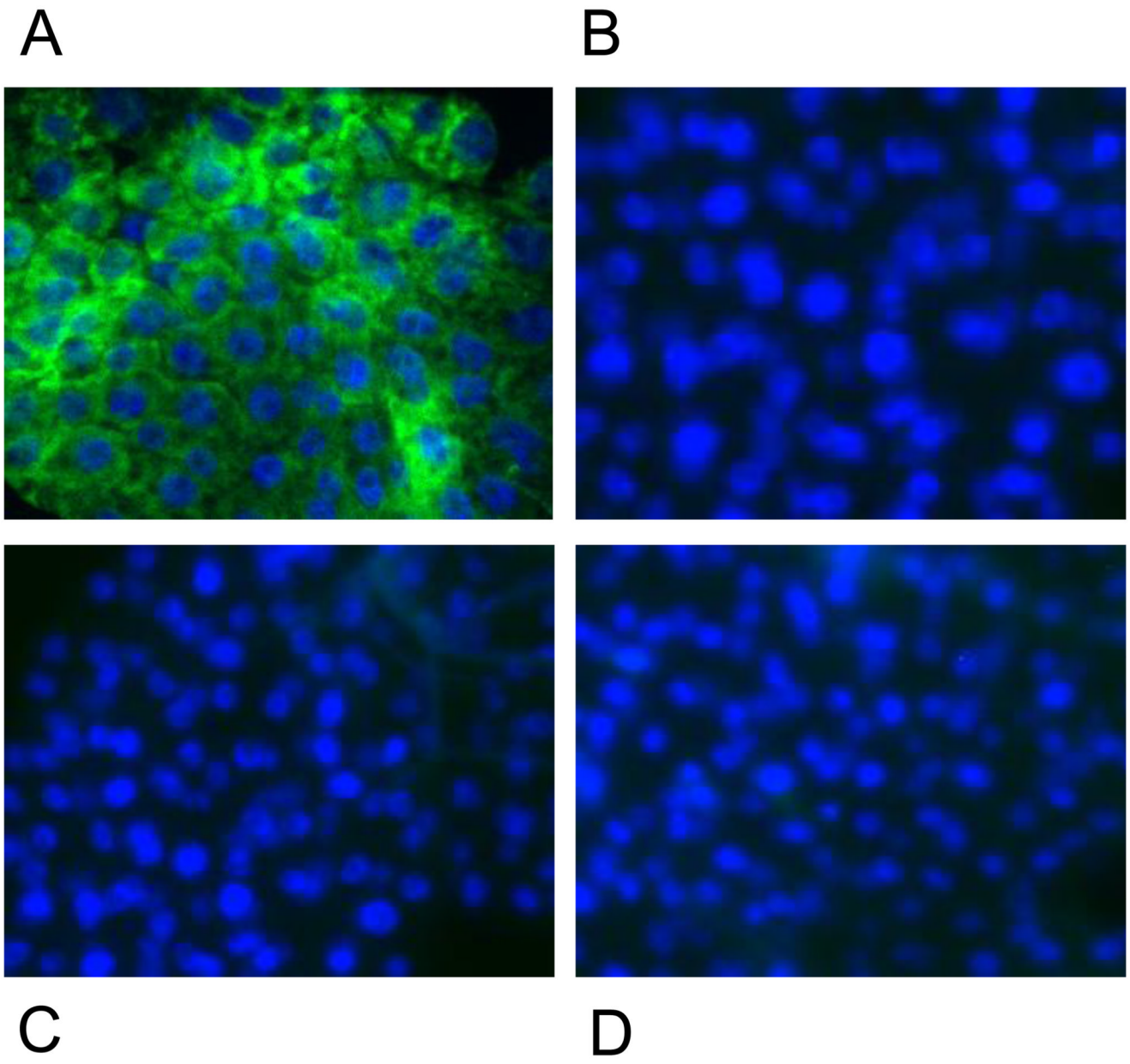

Fig. 8.

Comparison of EGFP reporter expression in fat bodies of transgenic and non-transgenic female mosquitoes after 20E activation in an *in vitro* organ culture. Specific EGFP expression was observed only in the *Vg-Gal4/UAS-EGFP* hybrids (A), but not in the *Vg-Gal4* driver (B), *UAS-EGFP* reporter (C), or non-transgenic UGAL females (D). Imaging was performed with a Zeiss AxioObserver A1 microscope using EGFP and Blue filters to obtain merged images of fat body preparations.