HHS Public Access

Author manuscript

Insect Biochem Mol Biol. Author manuscript; available in PMC 2015 November 01.

Published in final edited form as:

Insect Biochem Mol Biol. 2014 November ; 54: 1–10. doi:10.1016/j.ibmb.2014.08.001.

Regulation of the gut-specific carboxypeptidase: a study using the binary Gal4/UAS system in the mosquito *Aedes aegypti*

Bo Zhao[a,b], **Vladimir A. Kokoza**[a,d], **Tusar T. Saha**[a,d], **Stephanie Wang**[c], **Sourav Roy**[a,d], and **Alexander S. Raikhel**[a,d,*]

Bo Zhao: bzhao002@ucr.edu; Vladimir A. Kokoza: vladimir.kokoza@ucr.edu; Tusar T. Saha: tsaha001@ucr.edu; Stephanie Wang: swang024@ucr.edu; Sourav Roy: sourav.roy@ucr.edu

[a]Department of Entomology, University of California Riverside, Riverside, CA 92521, USA

[b]Graduate Program in Genetics, Genomics and Bioinformatics, University of California Riverside, Riverside, CA 92521, USA

[c]Honors Undergraduate Program, University of California Riverside, Riverside, CA 92521, USA

[d]The Institute for Integrative Genome Biology, University of California Riverside, Riverside, CA 92521, USA

Abstract

Pathogen transmission by mosquitoes is tightly linked to blood feeding which, in turn, is required for egg development. Studies of these processes would greatly benefit from genetic methods, such as the binary Gal4/UAS system. The latter has been well established for model organisms, but its availability is limited for mosquitoes. The objective of this study was to develop the blood-meal-activated, gut-specific Gal4/UAS system for the yellow-fever mosquito *Aedes aegypti* and utilize it to investigate the regulation of gut-specific gene expression. A 1.1-kb, 5' upstream region of the carboxypeptidase A (*CP*) gene was used to genetically engineer the CP-Gal4 driver mosquito line. The CP-Gal4 specifically activated the Enhanced Green Fluorescent Protein (EGFP) reporter only after blood feeding in the gut of the CP-Gal4>UAS-EGFP female *Ae. aegypti*. We used this system to study the regulation of *CP* gene expression. *In vitro* treatments with either amino acids (AAs) or insulin stimulated expression of the *CP-Gal4>UAS-EGFP* transgene; no effect was observed with 20-hydroxyecdysone (20E) treatments. The transgene activation by AAs and insulin was blocked by rapamycin, the inhibitor of the Target-of-Rapamycin kinase (TOR). RNA interference (RNAi) silence of the insulin receptor (IR) reduced the expression of the *CP-Gal4>UAS-EGFP* transgene. Thus, *in vitro* and *in vivo* experiments have revealed that insulin and TOR pathways control expression of the digestive enzyme CP. In contrast, 20E, the major regulator of post-blood-meal vitellogenic events in female mosquitoes, has no role in regulating the expression of this gene. This novel CP-Gal4/UAS system permits functional testing of midgut-

*Corresponding author: Alexander S. Raikhel; Department of Entomology and Institute for Integrative Genome Biology, University of California Riverside, Riverside, CA 92521, USA, Phone: +1 951 827 2129; alexander.raikhel@ucr.edu.

specific genes that are involved in blood digestion and interaction with pathogens in *Ae. aegypti* mosquitoes.

Keywords

Genetic transformation; Carboxypeptidase; Insulin; TOR; Mosquito; *Aedes aegypti*

1. Introduction

Female mosquitoes require vertebrate blood for egg development, and their cyclic feeding results in transmitting pathogens of numerous devastating human diseases. The yellow fever mosquito *Aedes aegypti* has become the predominant vector for the virus that causes Dengue fever, a life-threatening and debilitating disease, throughout many parts of the world. Understanding functions linked to blood feeding is essential for elucidating mechanisms of acquisition and transmission of disease pathogens. It could pave the way toward developing novel approaches for pathogen and vector control.

Significant progress has been made through genomic and post-genomic studies in mosquitoes. Genomes of three mosquito species—*Anopheles gambiae, Ae. aegypti* and *Culex quinquefasciatus*—have been sequenced, permitting identification of genes involved in mosquito-specific functions (Holt et al., 2002; Nene et al., 2007; Arensburger et al., 2010). However, hypotheses arising from genetic and genomic data need to be tested *in vivo* to elucidate the function of individual genes. Germ-line transformation allows introduction of such genes of interest into mosquitoes for identification of their functions. These transgenic studies in mosquitoes have been limited to direct overexpression of the gene of interest under the control of a selected promoter (Kokoza et al., 2000; Moreira et al., 2000; Adelman et al., 2008; Papathanos et al., 2009; Cho et al., 2006; Catteruccia et al., 2005; Nolan et al., 2011). Establishment of the Gal4/UAS system in model organisms has initiated a new era in elucidating gene functions (Brand and Perrimon, 1993; Ornitz et al., 1991; Scheer et al., 1999; Hartley et al., 2002; Imamura et al., 2003; Schinko et al., 2010). Essential genes, which when modified can have potentially harmful effects on development, behavior and fertility, can be investigated using the Gal4/UAS system. However, development of the Gal4/UAS system in mosquitoes has been slow, due to the difficulties in genetic transformation of these organisms and scarcity of available promoters with tissue/cell-, sex- and stage-specific expression. Kokoza and Raikhel established the first binary Gal4/UAS system for the female-, blood-meal-induced- and fat-body-specific expression in the mosquito *Ae. aegypti* (Kokoza and Raikhel, 2011). Lynd and Lycett have reported the midgut-specific Gal4/UAS system in the malaria vector *An. gambiae* (Lynd and Lycett, 2012). However, further refinement and development of the midgut-specific Gal4/UAS system is essential for characterization of regulatory mechanisms, governing expression of genes involved in blood digestion and pathogen interaction in mosquitoes. The goal of this work is to establish the midgut-specific Gal4/UAS system in *Ae. aegypti*.

In this study, we used the 5' upstream region of the *CP* gene to establish the *Ae. aegypti* CP-Gal4 driver line. We show that the CP-Gal4 driver activates a UAS-EGFP responder in

midguts of CP-Gal4>UAS-EGFP females in a blood meal dependent manner. Using this CP-Gal4/UAS system, we have investigated the regulation of transgene expression and shown it to be regulated by insulin and amino acid/TOR pathways. The development of the female midgut-specific CP-Gal4/UAS system for *Ae. aegypti* will enhance our ability to investigate genes involved in blood digestion. It will also be instrumental for studies of midgut factors that play a role in interactions with invading pathogens.

2. Materials and methods

2.1. Mosquito rearing

The *Ae. aegypti* UGAL/Rockefeller strain and transgenic lines were reared under identical laboratory conditions (27°C and 80% humidity) and kept in cages with unlimited access to 10% sugar solution and water until blood feeding. Three- to four-day-old female mosquitoes were blood-fed on White Leghorn chickens.

2.2. Plasmid construction

For construction of the pAehsp-pBac helper plasmid (Fig. S1), we used the 0.66-kb 5' regulatory region of the gene encoding the heat shock protein 70 (*Aehsp70*) (Isoe et al., 2007). PCR was used to amplify the *Aehsp70* fragment with genomic DNA from the *Ae. aegypti* UGAL/Rockefeller strain (wt) as template and *Aehsp70* gene-promoter-specific primers (Table S3). This fragment was incorporated into the *Sac*I-blunted site of the pBΔSac (Handler et al., 1998) to create pAehsp-pBac, placing the *Aehsp70* promoter upstream of the *piggyBac* transposase gene. The cloning strategy for the CP-Gal4 driver construct was as follows. Plasmid pBluescript-*AeCPA* promoter (Franz et al., 2006) was excised using *Sac*I and *Bam*HI, and the resulting 1.1-kb fragment was subcloned into the *Sma*I-*BamH*I site of the pSLfa1180fa shuttle vector (Horn and Wimmer, 2000) to generate pSL-CP. Then, a 0.8-kb *BamH*I-*Xba*I fragment of the chimeric Gal4 activator, excised from the pBac[3×P3-EGFP *afm*, *Vg-Gal4*] (Kokoza and Raikhel, 2011), was assembled into pSL-CP to form pSL-CP-Gal4. Adding a 0.25-kb *Not*I-*Afl*II SV40 terminator fragment into the pSL-CP-Gal4 produced the complete CP-Gal4 driver cassette. This resulting CP-Gal4 driver cassette was subcloned into the pBac [3×P3-EGFP *afm*] transformation vector (Horn and Wimmer, 2000) at the *Asc*I site to produce the CP-Gal4 driver construct.

2.3. Germ-line transformation of *Ae. aegypti*

Plasmid DNA used in injections was purified using the EndoFree Plasmid Maxi Kit (QIAGEN, Valencia, CA). CP-Gal4 driver (0.35mg/ml) and pAehsp-pBac helper (0.25mg/ml) plasmids were re-suspended in 0.1mM phosphate buffer (pH 6.8, containing 5mM KCl) and injected into the pre-blastoderm-stage eggs. The development of CP-Gal4 driver line was performed following a previously described process (Kokoza and Raikhel, 2011). G1 progeny were selected by monitoring the EGFP fluorescent eye marker under a Nikon SMZ800 fluorescence microscope fitted with GFP-B filter. The CP-Gal4>UAS-EGFP line was established as described previously (Kokoza and Raikhel, 2011), and the hybrid mosquitoes were selected by the presence of two eye-specific selectable markers, DsRed and EGFP. A Nikon SMZ800 fluorescence microscope fitted with DsRed and GFP-B filter sets was used for this selection.

2.4. Molecular analysis

Genomic DNA was extracted from adult mosquitoes using DNeasy tissue kit (QIAGEN, Valencia, CA). In genomic PCR reactions, 200 ng genomic DNA was used under conditions following the manufacturer's protocol for the Platinum PCR SuperMix (Invitrogen, Carlsbad, CA). Inverse PCR was performed on the M16-1 and F4-4 driver lines to investigate the insertion location in the genome. A total of 2 μg genomic DNA was digested with *Mbo*I and *Hpa*II (NEB, Ipswich, MA), and 1 μg of digested product was self-ligated in 400-μl total volume overnight at 16°C using T4 DNA Ligase (NEB, Ipswich, MA). The ligation product was purified and dissolved in 30 μl ddH2O. PCR was carried out to amplify DNA flanking the insertion locations using specific primers (Table S3), and the products were sequenced and verified against the VectorBase sequences, using Basic Local Alignment Search Tool (BLAST).

For each experimental treatment, total RNA was extracted from 10 midguts using Trizol (Invitrogen, Carlsbad, CA). cDNAs were synthesized from 2 μg of total RNA after DNaseI (Invitrogen, Carlsbad, CA) treatment, using Omniscript Reverse transcriptase kit (QIAGEN, Valencia, CA) in 20 μl of the reaction mixture. RT-PCR and qRT-PCR for selected transcripts were done and analyzed as described previously (Kokoza and Raikhel, 2011; Roy et al., 2007).

2.5. Immunofluorescence

10 midguts from each treatment were fixed at room temperature for 20 min in 3.7% (w/v) formaldehyde in phosphate buffered saline (PBS). Fixed midguts were washed (three times, 5 min each) in PBS-T, which contained 0.3% Triton X-100 (v/v) in PBS, before being blocked for 1 h in 3% (w/v) bovine serum albumin in PBS-T at room temperature. After rinsing in PBS-T, midguts were incubated at 4°C overnight with anti-GFP antibodies diluted 1:200 in PBS-T (polyclonal, developed in rabbit, Sigma-Aldrich, St. Louis, MO). Midguts were washed and incubated with Fluorescein-conjugated Anti-Rabbit IgG (Vector, Burlingame, CA) diluted 1:400 in PBS-T for 2 h at room temperature, then mounted using VECTASHIELD Mounting Medium (Vector, Burlingame, CA). Images were captured and analyzed under a Leica M165 FC fluorescent stereo microscope and using LAS V4.0 software. Photographs are representative of ten mosquitoes for each treatment.

2.6. *In vitro* midgut culture

The *in vitro* organ culture was performed as previously described for the fat body (Roy et al., 2007). Tissue was incubated either in culture medium lacking AAs (AA−; containing equimolar amounts of mannitol in place of AAs) or with medium containing all the AAs (AA+) for 8 h at 27°C (1 midgut/60 μl/dish). In addition, the following reagents were used: bovine insulin at a final concentration of 17 μM (Sigma-Aldrich, St. Louis, MO, USA), 20 pmol rapamycin (LC Laboratory; dissolved in ethanol) and 20E at the final concentration of 10 μM (Sigma-Aldrich, St. Louis, MO, USA; dissolved in ethanol). Midguts were dissected from 3-day-old, non-blood-fed females.

2.7. RNA interference

Double-stranded RNA (dsRNA) was produced using the MEGAscript T7 kit (Ambion, Austin, TX, USA), and a total of 2 μg in 0.3–0.5 μl distilled water was injected into 1-day-old female mosquitoes. These mosquitoes were maintained on sucrose solution for 4 days, then given a blood meal, after which their midguts were isolated (at 24 h PBM). Transcript abundance was analyzed using qRT-PCR analysis as described above. RNAi depletion of luciferase (dsLuc) served as a control.

3. Results

3.1. Establishment of CP-Gal4 transgenic lines

piggyBac transposable element was utilized to create the transgenic *Ae. aegypti* CP-Gal4 driver plasmid (Fig. 1A). This CP-Gal4 driver plasmid contained the 1.1-kb 5' upstream region of the *CP* gene that was linked to a modified yeast Gal4 protein coding sequence (Kokoza and Raikhel, 2011; Kulkarni and Arnosti, 2003), followed by a SV40 polyadenylation signal. The CP-Gal4 driver cassette contained a selectable marker, consisting of the *EGFP* gene driven by the eye-specific *3×P3* gene promoter. To construct the *piggyBac* helper plasmid, we employed the 0.66-kb 5' upstream promoter region of the *Ae. aegypti* heat shock protein70 gene (*Aehsp70*) (Isoe et al., 2007) instead of the previously used *Drosophila melanogaster hsp70* gene (Handler and Harrell II, 1999) (Fig. S1).

The pre-blastoderm embryos of the UGAL/Rockefeller *Ae. aegypti* strain (wt) were injected with a mixture of the CP-Gal4 driver and helper constructs. Of 1084 injected embryos, 90 survived to adulthood. These G0 mosquitoes were separated by sex with 43 males and 47 females. Each G0 male was out-crossed individually with 5 wt females giving rise to 43 male families (M1-M43). 47 females were separated into 6 groups. In each of these six G0 group, females were out-crossed with an equal number of wt males, forming 6 female families (F1–F6). G1 progeny (larvae and pupae) were examined for the presence of EGFP expression in their eyes. Based on this criterion, two individuals were selected, M16-1 from M16 male family and F4-4 from F4 female family. Their progeny was established as homozygous based on the selection for 100% eye-specific EGFP expression of mosquitoes in each line. Pupae of the M16-1 line showed much stronger eye-specific marker gene expression than those of the F4-4 line (Fig. 1B–C). Results indicated that the transformation efficiency was 2.2% (Table S1).

Genome integration of the CP-Gal4 construct in the M16-1 and F4-4 driver lines was confirmed by means of genomic polymerase chain reaction (PCR) using specific primers for the *piggyBac* transposon left and right arms, as well as the CP-Gal4 sequence (Fig. 1E). The UAS-EGFP sequence was used in these PCR analyses as a control. While *piggyBac* transposon sequences (left arm and right arm) were detected in the driver and responder transgenic lines, the CP-Gal4 sequence was only present in the genomic DNA isolated from the CP-Gal4 driver lines, M16-1 and F4-4. DNA from the UAS-EGFP responder line contained UAS-EGFP responder sequence that was not present in either the driver line or wt mosquitoes (Fig. 1E).

Genome incorporation of the *CP-Gal4* transgene (*CPT*) was determined by means of inverse PCR analysis using genomic DNA from M16-1 and F4-4 transgenic lines. Insertion of the *piggyBac* transposon with canonical TTAA target sites containing the *CPT* was found in M16-1 and F4-4 transgenic lines (Table S2). The insertion sites were located outside of predicted gene coding sequences (VectorBase AaegL 1.3) in both transgenic lines. The UAS-EGFP responder line with a DsRed selectable marker has been generated and characterized previously (Kokoza and Raikhel, 2011).

The pattern of *CPT* expression in M16-1 and F4-4 transgenic lines was investigated using RNA isolated from the digestive system (midgut) of female mosquitoes from the wt, UAS-EGFP responder, M16-1 and F4-4 transgenic driver lines at 72 h post-eclosion, a time point during the previtellogenic (PV) stage before blood feeding and several time points after blood feeding (Fig. 2A–D). This analysis was accomplished by means of the reverse-transcriptase polymerase chain reaction (RT-PCR), using primers specific to the endogenous *CP*, transgenic *CPT* and *UAS-EGFP* (Table S3). There was no expression of the *UAS-EGFP* transgene in midguts of any of the tested mosquito lines (Fig. 2A–D). The expression pattern of the *CP* gene was similar in all four tested mosquito lines; the CP transcript was barely visible before blood feeding, it increased PBM, reaching the peak at 24 h PBM, and decreased thereafter (Fig. 2A–D). In the M16-1 line, *CPT* expression followed a temporal pattern similar to that of the endogenous *CP* gene (Fig. 2C). However, in the F4-4 line, the *CPT* was expressed during the previtellogenic stage and peaked at 12 h PBM (Fig. 2D). Because the *CPT* expression pattern in the F4-4 line did not match that of the endogenous *CP*, the M16-1 CP-Gal4 driver line was chosen for further analysis. Expression of *CPT* was not observed in the wt or the UAS-EGFP responder lines.

3.2. Development of CP-Gal4>UAS-EGFP hybrid mosquitoes

The CP-Gal4>UAS-EGFP hybrid mosquitoes were produced by crossing the M16-1 CP-Gal4 homozygous driver line with the UAS-EGFP homozygous responder line. These CP-Gal4>UAS-EGFP hybrid mosquitoes exhibited the presence of both EGFP and DsRed eye-specific markers (Fig. 3A–D). Genomic PCR analysis confirmed the presence of *piggyBac* transposon left arm, right arm, CP-Gal4 driver and UAS-EGFP responder sequences in the hybrid mosquitoes (Fig. 3E).

In the CP-Gal4>UAS-EGFP hybrid female mosquitoes, the *EGFP* transgene expression followed a pattern similar to that of the endogenous *CP* and *CPT* (Fig. 4A). Expression of the *UAS-EGFP* transcript was midgut-specific and was not found in the fat body (FB), ovaries (OV) or Malpighian tubules (MT) of hybrid female mosquitoes 24 h PBM (Fig. 4B). Males from this hybrid line did not express *UAS-EGFP* (Fig. 4B). These data indicated that, in the CP-Gal4>UAS-EGFP mosquitoes, the *UAS-EGFP* transgene exhibited a tissue-, sex-, and stage-specific expression pattern similar to that of the endogenous *CP* gene.

To assess the EGFP protein level in the CP-Gal4>UAS-EGFP mosquitoes, we dissected and examined midgut with a fluorescence microscope. This analysis showed a strong signal in midguts of CP-Gal4>UAS-EGFP female mosquitoes at 24 h PBM. In contrast, only background signal was observed in midguts from the CP-Gal4 driver, UAS-EGFP responder lines and wt mosquitoes examined at the same time (Fig. 4C). This confirmed that the EGFP

protein was produced in the CP-Gal4>UAS-EGFP female mosquitoes only after blood-meal activation.

3.3. Insulin and amino acid/TOR signaling pathways are involved in activation of *CP-Gal4>UAS-EGFP* transgene expression

To elucidate the signals involved in regulation of *CP-Gal4>UAS-EGFP* transgene expression, we first used an *in vitro* organ culture assay (Fig. 5). Digestive systems (midgut) isolated from the CP-Gal4>UAS-EGFP transgenic female mosquitoes were incubated in the presence or absence of specific factors in a culture medium. Results were evaluated by means of quantitative reverse-transcriptase PCR (qRT-PCR), using primers for the *CP-Gal4* (Gal4) and *UAS-EGFP* (EGFP) transgenes (Table S3). The tissue incubated in the medium without AAs showed a low, baseline level expression of the *CP-Gal4>UAS-EGFP* transgene transcript, while addition of AAs to the medium resulted in a statistically significant increase in transcript levels (Fig. 5A). Insulin alone did not increase the *CP-Gal4>UAS-EGFP* transgene transcript level; however, the transcript level rose significantly after incubation in medium containing both AAs and insulin together. Testing expression of both the *CP-Gal4* and the *UAS-EGFP* transgene has permitted us to demonstrate that their expression is clearly co-regulated.

When we examined the EGFP protein level using immunocytochemistry, we observed a substantial increase in the signal only after incubation of midguts from CP-Gal4>UAS-EGFP hybrid female mosquitoes in the medium supplemented with AAs and insulin (Fig. 5B). Thus, *in vitro* organ culture experiments have shown that both insulin and AAs are required for transcription and translation of the CP-Gal4-driven EGFP in the midgut of the transgenic mosquitoes. Moreover, they exhibit a strong additive effect.

To substantiate *in vitro* experiments, we investigated the *in vivo* effect of RNAi depletions of potential factors involved in regulation of the *CP-Gal4>UAS-EGFP* transgene in the mosquito midgut. Because insulin has been identified as a principal regulator of the transgene expression, we examined the effect of RNAi depletion of the InR. There is a single gene encoding InR in the *Ae. aegypti* genome (Brown et al., 2008). RNAi depletion of InR *in vivo* resulted in a significant decrease of the *CP-Gal4>UAS-EGFP* transgene transcript level in midguts at 24h PBM, as indicated by testing with Gal4 and EGFP probes (Fig. 6A). To confirm that the effect observed after InR RNAi depletions correlated with the responsiveness to insulin, we incubated midguts isolated from the non-blood fed *CP-Gal4>UAS-EGFP* transgenic female mosquitoes at 72 h post-eclosion treated with Luciferase double-stranded RNA (dsLuc) or double-stranded insulin receptor RNA (dsInR) in the amino acid-containing culture media in the presence or absence of insulin (Fig. 6B–C). Addition of insulin elevated the *CP-Gal4>UAS-EGFP* transgene transcript in the dsLuc-treated mosquitoes. In contrast, no response to insulin was observed in dsInR-treated mosquitoes (Fig. 6B–C). Thus, *in vivo* RNAi experiment results were in agreement with those from the *in vitro* tissue culture, strongly suggesting that insulin plays a major role in regulating *CP* gene expression.

Target of Rapamycin kinase (TOR) is a conserved nutritional sensor that mediates both insulin and AA signaling (Grewal, 2008). We tested whether TOR inhibition affects the

expression of the *CP-Gal4>UAS-EGFP* transgene transcript. We injected 3-day-old, *CP-Gal4>UAS-EGFP* female mosquitoes with the TOR inhibitor rapamycin at different concentrations and examined at 24 h PBM. Results indicated that the transgene transcript expression was inhibited by rapamycin in a dose-dependent manner (Fig. S3A), and rapamycin did not affect survival of mosquitoes (Fig. S3B). Rapamycin significantly decreased the transcript level of this gene in the *CP-Gal4>UAS-EGFP* female mosquitoes treated with dsLuc (Fig. 7A). RNAi depletion of InR significantly decreased the level of the *CP-Gal4>UAS-EGFP* transgene transcript when compared with that of the dsLuc. However, this transcript level was completely diminished in transgenic mosquitoes co-treated with rapamycin and dsInR (Fig. 7A). Rapamycin also prevented elevation of this transcript by insulin in the mosquito digestive system in an i*n vitro* culture assay (Fig. 7B).

20E is a major regulator of the PBM reproductive events; however, its role in regulating digestive enzymes is not understood. To investigate the possible influence of the ecdysone regulatory cascade on the *CP-Gal4>UAS-EGFP* transgene expression, we first investigated the effect of 20E using an *in vitro* organ culture assay, as described above. Supplementing the medium with 20E did not elevate its level, regardless of the presence or absence of AAs (Fig. 5A). Next, we used *in vivo* ecdysone receptor (EcR) RNAi depletion. The double-stranded RNA for EcR (dsEcR) was designed using the region common to both EcR-A and EcR-B isoforms. This depletion had no effect on the transgene transcript level, which was similar to that of the dsLuc control (Fig. 8). The dsEcR treatments also had no effect on the transcript abundance of the endogenous *CP* gene examined in parallel with that of the *CP-Gal4>UAS-EGFP* transgene transcript at 24 h and 36 h PBM *in vivo* (Fig. 8).

Analysis of the *CP* gene promoter has revealed the presence of a putative binding site—tgtttag—for Broad isoform Z4 (Br-Z4). *Br* is a downstream gene in the 20E hierarchy, although it is reportedly not entirely dependent on 20E signaling and can act independently (Brennan et al., 2001; Gancz et al., 2011). In our experiments, EcR RNAi depletion significantly reduced the level of Br-Z4 transcript, suggesting that this gene is under the control of 20E hierarchy (Fig. 8B). However, to further investigate a possible involvement of Br-Z4 in regulation of the *CP-Gal4>UAS-EGFP* transgene, we conducted Br-Z4 RNAi depletion. The double-stranded RNA of Br-Z4 (dsBr-Z4) treatment did not affect the transcript abundance of the transgene gene examined at 24 h and 36 h PBM *in vivo* (Fig. 8C). It is possible that Br-Z4 plays an important role in regulation of *CP* gene expression during other stages of the mosquito life cycle. Effects of RNAi depletions of tested factors on their own transcript levels are presented in Figure S2.

Thus, our study using the *CP-Gal4>UAS-EGFP* transgene has shown that insulin and AA/TOR signaling pathways, but not 20E, are involved in regulating the expression of the gene encoding the late-phase blood digestive enzyme CP.

4. Discussion

In this study, we expanded the development of the binary Gal4/UAS system for the yellow fever mosquito *Ae. aegypti* and used the 5' upstream region of the *CP* gene, which is induced by a blood meal in the midgut of female mosquitoes (Isoe et al, 2009a; Edwards et al.,

2000), to genetically engineer the transgenic CP-Gal4 driver line. After a blood meal, the *CP* gene expression and enzymatic activity of CP increases rapidly and reaches its peak at 24 h (Edwards et al., 2000; Noriega et al., 2002). The 1.1-kb 5' upstream region of the *CP* gene is sufficient to lead transgene expression with a profile similar to that of the endogenous *CP* gene (Franz et al., 2006). The important feature of this driver is that the *CPT* exhibits specific expression in the female mosquito midgut, and is activated by blood feeding. We generated two *Ae. aegypti* CP-Gal4 driver lines. However, only the M16-1 line expressed the transgene in a manner consistent with that of the endogenous *CP*, from which the regulatory DNA was derived. In contrast, the F4-4 line showed a different transgene expression profile, in which the *CPT* transcript was expressed prior to blood-meal activation and reached its peak at 12 h PBM rather than 24 h. The integration transgene site for F4-4 located 8 kb upstream of a predicted gene, AAEL004671, which encodes (s)-2-hydroxy-acid oxidase. The expression pattern in the F4-4 line was possibly affected by its integration location. Transgene miss-expression has previously been reported for midgut-specific transgenes in *An. gambiae* and *Ae. aegypti* (Lynd and Lycett, 2012; Franz et al., 2011). Integration of a transgene in a specific site in the genome is critical for its correct expression. For example, integration near a certain transcription enhancer or repressor could lead to an unintended transgene expression pattern (Wallrath and Elgin, 1995), and integration near a transcription repressor or heterochromatic region could result in low levels of transgene expression (O'Kane and Gehring, 1987). In some transposable-element-generated mosquito lines, transgenes have reportedly been poorly expressed because of the position effect (Nimmo et al., 2006; Labbe et al., 2010). Franz et al. described that when the *CP* gene promoter region was used to drive EGFP reporter expression in *Ae. aegypti*, only two lines from a total number of nine transgenic mosquitoes lines robustly expressed the EGFP reporter (Franz et al., 2011). Lycett et al. (2012) found that the alpha-tubulin-1b gene promoter directed transgene expression in the head, ventral nerve cord and testes of one *An. gambiae* transgenic line; however, expression of the same transgene was observed in larval and adult muscles, fat body, cuticle and midgut secretory cells of a different transgenic line. The lack of more advanced techniques for mosquito transformation similar to those of Drosophila complicates a challenging task of mosquito genetic transformation.

Multiple enzymes are involved in blood digestion in the midgut of female mosquitoes. These include trypsin-like and chymotrypsin-like serine endoproteases, amino- and carboxypeptidases (Isoe et al., 2009a; Isoe et al., 2009b). In *Ae. aegypti*, mRNA of the early trypsin (*AaET*) gene is expressed and accumulated in the midgut cells prior to blood feeding, while the synthesis of the AaET protein occurs just after blood feeding (Noriega et al., 1996). The transcript and protein levels of a digestive endoprotease, late trypsin (AaLT), reach their peaks by 24 h post blood meal (PBM) (Isoe et al., 2009b; Barillas-Mury et al., 1993). Recent studies have revealed seven additional midgut serine proteases (AaSPs); the expression profiles of genes encoding these proteases are similar to that of the *AaLT* gene (Isoe et al., 2009a; Brackney et al., 2010). The RNAi and enzymatic analyses have demonstrated differential roles of midgut serine proteases. Knockdown of AaSPVI caused a 78% decrease in the late-phase, trypsin-like activity, while knockdowns of AaLT, AaSPVI and AaSPVII inhibited degradation of the serum albumin (Isoe et al., 2009a). Carboxypeptidases represent another important group of mosquito gut proteases (Isoe et al,

2009b). The gene encoding *Ae. aegypti* carboxypeptidase A has an expression profile similar to that of the *AaLT* gene (Edwards et al., 2000). However, the regulation of the *CP* gene has not been studied.

Previous studies on amino acids suggest that these blood components serve as nutritional signal in digestive protease introduction (Caroci and Noriega, 2003). Amino acids are key stimuli, which play a role in regulating the translation of AaET (Brandon et al., 2008). Eight insulin-like peptides (ILPs) have been reported in *Ae. aegypti*, among which AaILP3 binds the InR (Riehle et al., 2006; Wen et al., 2010). Gulia-Nuss et al. reported that amino acids and insulin signaling pathways regulate *Ae. aegypti* late phase gene expression, blood digestion and egg maturation (Gulia-Nuss et al., 2011). InR RNAi knockdown or TOR inhibition reduces the *AaLT* gene expression and the activity of this enzyme (Gulia-Nuss et al., 2011).

Using the *Ae. aegypti* CP-Gal4>UAS-EGFP line with the M16- 1 Gal4 driver, we established that the expression of the *UAS-EGFP* transgene matched that of the endogenous *CP* gene as well as that of the *CPT*. *In vitro* organ-culture experiments demonstrated a synergistic action of insulin and AAs in activating expression of the *CP-Gal4>UAS-EGFP* transgene. These observations were in agreement with *in vivo* RNAi depletion experiments. RNAi silencing of InR resulted in a significant decrease in the *CP-Gal4>UAS-EGFP* transgene transcript level. Application of rapamycin, a specific inhibitor of TOR, had a strong inhibitory effect on the *CP-Gal4>UAS-EGFP* transgene transcript expression, indicating importance of the TOR kinase in regulation of the *CP* gene. Moreover, the transcript level was completely diminished in transgenic mosquitoes co-treated with rapamycin and dsInR. This inhibition was significantly stronger than that caused by InR RNAi depletion alone, suggesting additive effects of TOR and insulin. Treatment with rapamycin also prevented elevation of this transcript after incubation with either AAs or insulin *in vitro*, confirming independent effects of these regulators. The TOR kinase pathway has been reported to integrate extracellular signals such as insulin and AAs (Nave et al. 1999; Sarbassov et al. 2005). Thus, insulin and AA/TOR signaling pathways are involved in regulating expression of the *CP* gene. Taken together, our investigation along with previous studies has revealed important roles of insulin and nutrition-driven AA/TOR pathways in regulating the expression of late-phase digestive enzymes in the mosquito midgut.

20E is the major regulator of vitellogenic events in female mosquitoes (Raikhel et al., 2005). It regulates expression of genes encoding yolk protein genes in the fat body. However, our experiments revealed no apparent role of this hormone in controlling the *CP* gene in the female mosquito midgut. The level of the *CP-Gal4>UAS-EGFP* transgene transcript was not affected as a result of *in vitro* midgut incubations in the presence of 20E. Moreover, EcR RNAi silencing had no effect on the transcript level of this transgene. The absence of ecdysone response elements (EcRE) or binding sites for any other known 20E downstream factors (other than that of BrZ4) within the *CP* gene promoter further corroborates these results. RNAi silencing has shown that BrZ4 is not involved in regulation of the *CP* gene in the gut of female mosquitoes. Thus, the presence of the BrZ4 recognition site in the *CP*

promoter could be explained by a possible contribution of this transcription factor in control of *CP* gene expression during other stages of development.

In conclusion, this study contributes toward the improvement of genetics tools for mosquitoes. We developed an *Ae. aegypti* CP-Gal4 driver line that is capable of directing a female, midgut-specific and blood-meal-induced expression of the UAS/target transgene. Using the CP-Gal4>UAS-EGFP system, we have demonstrated that activation of the *CP* gene depends on insulin/InR and nutrient-driven AA/TOR pathways. In contrast, 20E, which is the major regulator of mosquito vitellogenesis, plays no role in *CP* gene activation. Establishment of the *Ae. aegypti* CP-Gal4/UAS system permits functional testing of various midgut-specific genes involved in blood digestion and pathogen interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Kenneth E. Olson for providing the pBluescript-*AeCPA* promoter plasmid. This work was supported by NIH grant RO1 AI113729.

Abbreviations

CP	carboxypeptidase A
EGFP	enhanced green fluorescent protein
AAs	amino acids
20E	20-hydroxyecdysone
TOR	Target-of-Rapamycin
RNAi	RNA interference
InR	insulin receptor
MG	midgut
PBM	post blood meal
WT	wild type
PCR	polymerase chain reactions
CPT	CP-Gal4 transgene
PV	previtellogenic
RT-PCR	reverse-transcriptase polymerase chain reaction
FB	fat body
OV	ovaries
MT	Malpighian tubules

qRT-PCR	quantitative reverse transcriptase PCR
dsLuc	double-stranded RNA of Luciferase
dsInR	double-stranded RNA of insulin receptor
EcR	Ecdysone Receptor
dsEcR	double-stranded RNA of Ecdysone Receptor
Br-Z4	Broad isoform Z4
dsBr-Z4	double-stranded RNA of Br-Z4
EcRE	ecdysone response element
ILPs	insulin-like peptides
BLAST	basic local alignment search tool
PBS	Phosphate Buffered Saline
dsRNA	double-stranded RNA

References

Adelman ZN, Anderson MA, Morazzani EM, Myles KM. A transgenic sensor strain for monitoring the RNAi pathway in the yellow fever mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol. 2008; 38:705–713. [PubMed: 18549956]

Arensburger P, Megy K, Waterhouse RM, Abrudan J, Amedeo P, Antelo B, Bartholomay L, Bidwell S, Caler E, Camara F, Campbell CL, Campbell KS, Casola C, Castro MT, Chandramouliswaran I, Chapman SB, Christley S, Costas J, Eisenstadt E, Feschotte C, Fraser-Liggett C, Guigo R, Haas B, Hammond M, Hansson BS, Hemingway J, Hill SR, Howarth C, Ignell R, Kennedy RC, Kodira CD, Lobo NF, Mao C, Mayhew G, Michel K, Mori A, Liu N, Naveira H, Nene V, Nguyen N, Pearson MD, Pritham EJ, Puiu D, Qi Y, Ranson H, Ribeiro JM, Roberston HM, Severson DW, Shumway M, Stanke M, Strausberg RL, Sun C, Sutton G, Tu ZJ, Tubio JM, Unger MF, Vanlandingham DL, Vilella AJ, White O, White JR, Wondji CS, Wortman J, Zdobnov EM, Birren B, Christensen BM, Collins FH, Cornel A, Dimopoulos G, Hannick LI, Higgs S, Lanzaro GC, Lawson D, Lee NH, Muskavitch MA, Raikhel AS, Atkinson PW. Sequencing of *Culex quinquefasciatus* establishes a platform for mosquito comparative genomics. Science. 2010; 330:86–88. [PubMed: 20929810]

Barillas-Mury CV, Wells MA. Cloning and sequencing of the blood meal-induced late trypsin gene from the mosquito *Aedes aegypti* and characterization of the upstream regulatory region. Insect Mol. Bio. 1993; 2:7–12. [PubMed: 9087537]

Brackney DE, Isoe J, Black WCIV, Zamora J, Foy BD, Miesfeld RL, Olson KE. Expression profiling and comparative analyses of seven midgut serine proteases from the yellow fever mosquito, *Aedes aegypti*. J. Insect Physiol. 2010; 56:736–744. [PubMed: 20100490]

Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993; 118:401–415. [PubMed: 8223268]

Brandon MC, Pennington JE, Isoe J, Zamora J, Schillinger AS, Miesfeld RL. TOR signaling is required for amino acid stimulation of early trypsin protein synthesis in the midgut of *Aedes aegypti* mosquitoes. Insect Biochem. Mol. Biol. 2008; 38(10):916–922. [PubMed: 18708143]

Brennan CA, Li TR, Bender M, Hsiung F, Moses K. Broad-complex, but not ecdysone receptor, is required for progression of the morphogenetic furrow in the Drosophila eye. Development. 2001; 128:1–11. [PubMed: 11092806]

Brown MR, Clark KD, Gulia M, Zhao Z, Garczynski SF, Crim JW, Suderman RJ, Strand MR. An insulin-like peptide regulates egg maturation and metabolism in the mosquito *Aedes aegypti*. Proc. Natl. Acad. Sci. USA. 2008; 105:5716–5721. [PubMed: 18391205]

Caroci SA, Noriega GF. Free amino acids are important for the retention of protein and non-protein meals by the midgut of *Aedes aegypti* females. J Insect Physiol. 2003; 49(9):839–844. [PubMed: 16256686]

Catteruccia F, Benton JP, Crisanti A. An Anopheles transgenic sexing strain for vector control. Nat. Biotechnol. 2005; 23:1414–1417. [PubMed: 16244659]

Cho KH, Cheon HM, Kokoza V, Raikhel AS. Regulatory region of the vitellogenin receptor gene sufficient for high-level, germ line cell-specific ovarian expression in transgenic *Aedes aegypti* mosquitoes. Insect Biochem. Mol. Biol. 2006; 36:273–281. [PubMed: 16551541]

Edwards MJ, Moskalyk LA, Donelly-Doman M, Vlaskova M, Noriega FG, Walker VK, Jacobs-Lorena M. Characterization of a carboxypeptidase A gene from the mosquito, *Aedes aegypti*. Insect Mol. Biol. 2000; 9:33–38. [PubMed: 10672069]

Franz AW, Sanchez-Vargas I, Adelman ZN, Blair CD, Beaty BJ, James AA, Olson KE. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. Proc. Natl. Acad. Sci. USA. 2006; 103:4198–4203. [PubMed: 16537508]

Franz AW, Jasinskiene N, Sanchez-Vargas I, Isaacs AT, Smith MR, Khoo CCH, Heersink MS, James AA, Olson KE. Comparison of transgene expression in *Aedes aegypti* generated by mariner *Mos 1* transposition and *ΦC31* site-directed recombination. Insect Mol. Biol. 2011; 20:587–598. [PubMed: 21699593]

Gancz D, Lengil T, Gilboa L. Coordinated regulation of niche and stem cell precursors by hormone signaling. Plos Biology. 2011; 9:e1001202. [PubMed: 22131903]

Grewal SS. Insulin/TOR signaling in growth and homeostasis: A view from the fly world. Int. J. Biochem. Cell Biol. 2008; 41(5):1006–1010. [PubMed: 18992839]

Gulia-Nuss M, Robertson AE, Brown MR, Strand MR. Insulin-like peptides and the Target of Rapamycin pathway coordinately regulate blood digestion and egg maturation in the mosquito *Aedes aegypti*. Plos One. 2011; 6(5):e20401. [PubMed: 21647424]

Handler AM, Harrell RA II. Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Mol. Biol. 1999; 8:449–457. [PubMed: 10634970]

Handler AM, McCombs SD, Fraser MJ, Saul SH. The lepidopteran transposon vector, *piggyBac* mediates germ-line transformation in the Mediterranean fruit fly. Proc. Natl. Acad. Sci. USA. 1998; 95:7520–7525. [PubMed: 9636182]

Hartley KO, Nutt SL, Amaya E. Targeted gene expression in transgenic Xenopus using the binary Gal4/UAS system. Proc. Natl. Acad. Sci. USA. 2002; 99:1377–1382. [PubMed: 11818539]

Holt RA, Subramanian GM, Halpern A, Sutton GG, Charlab R, Nusskern DR, Wincker P, Clark AG, Ribeiro JM, Wides R, Salzberg SL, Loftus B, Yandell M, Majoros WH, Rusch DB, Lai Z, Kraft CL, Abril JF, Anthouard V, Arensburger P, Atkinson PW, Baden H, de Berardinis V, Baldwin D, Benes V, Biedler J, Blass C, Bolanos R, Boscus D, Barnstead M, Cai S, Center A, Chaturverdi K, Christophides GK, Chrystal MA, Clamp M, Cravchik A, Curwen V, Dana A, Delcher A, Dew I, Evans CA, Flanigan M, Grundschober-Freimoser A, Friedli L, Gu Z, Guan P, Guigo R, Hillenmeyer ME, Hladun SL, Hogan JR, Hong YS, Hoover J, Jaillon O, Ke Z, Kodira C, Kokoza E, Koutsos A, Letunic I, Levitsky A, Liang Y, Lin JJ, Lobo NF, Lopez JR, Malek JA, McIntosh TC, Meister S, Miller J, Mobarry C, Mongin E, Murphy SD, O'Brochta DA, Pfannkoch C, Qi R, Regier MA, Remington K, Shao H, Sharakhova MV, Sitter CD, Shetty J, Smith TJ, Strong R, Sun J, Thomasova D, Ton LQ, Topalis P, Tu Z, Unger MF, Walenz B, Wang A, Wang J, Wang M, Wang X, Woodford KJ, Wortman JR, Wu M, Yao A, Zdobnov EM, Zhang H, Zhao Q, Zhao S, Zhu SC, Zhimulev I, Coluzzi M, della Torre A, Roth CW, Louis C, Kalush F, Mural RJ, Myers EW, Adams MD, Smith HO, Broder S, Gardner MJ, Fraser CM, Birney E, Bork P, Brey PT, Venter JC, Weissenbach J, Kafatos FC, Collins FH, Hoffman SL. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science. 2002; 298:129–149. [PubMed: 12364791]

Horn C, Wimmer EA. A versatile vector set for animal transgenesis. Dev. Genes Evol. 2000; 210:630–637. [PubMed: 11151300]

Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, Tamura T. Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. Genetics. 2003; 165:1329–1340. [PubMed: 14668386]

Isoe J, Kunz S, Manhart C, Wells MA, Miesfeld RL. Regulated expression of microinjected DNA in adult *Aedes aegypti* mosquitoes. Insect Mol. Biol. 2007; 16:83–92. [PubMed: 17257211]

Isoe J, Rascón AA Jr, Kunz S, Miesfeld RL. Molecular genetic analysis of midgut serine proteases in *Aedes aegypti* mosquitoes. Insect Biochem. Mol. Biol. 2009a; 39:903–912. [PubMed: 19883761]

Isoe J, Zamora J, Miesfeld RL. Molecular analysis of the *Aedes aegypti* carboxypeptidase gene family. Insect Biochem. Mol. Biol. 2009b; 39:68–73. [PubMed: 18977440]

Kokoza V, Ahmed A, Cho WL, Jasinskiene N, James AA, Raikhel AS. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. Proc. Natl. Acad. Sci. USA. 2000; 97:9144–9149. [PubMed: 10908672]

Kokoza V, Raikhel AS. Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. Insect Biochem. Mol. Biol. 2011; 41:637–644. [PubMed: 21536128]

Kulkarni M, Arnosti DN. Information display by transcriptional enhancers. Development. 2003; 130:6569–6575. [PubMed: 14660545]

Labbe GMC, Nimmo DD, Alphey L. *piggybac*- and *ΦC31*-mediated genetic transformation of the Asian tiger mosquito, *Ae. albopictus* (Skuse). PLoS Negl. Trop. Dis. 2010; 4(8):e788. [PubMed: 20808959]

Lycett GJ, Amenya D, Lynd A. The *Anopheles gambiae* alpha-tubulin-1b promoter directs neuronal, testes and developing imaginal tissue specific expression and is a sensitive enhancer detector. Insect Mol. Biol. 2012; 21:79–88. [PubMed: 22011081]

Lynd A, Lycett GJ. Development of the Bi-Partite Gal4-UAS System in the African Malaria Mosquito, *Anopheles gambiae*. PLoS ONE. 2012; 7(2):e31552. [PubMed: 22348104]

Moreira LA, Edwards MJ, Adhami F, Jasinskiene N, James AA, Jacobs-Lorena M. Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes. Proc. Natl. Acad. Sci. USA. 2000; 97:10895–10898. [PubMed: 11005862]

Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J. 1999; 334(2):427–431. [PubMed: 10567225]

Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, Debruyn B, Decaprio D, Eiglmeier K, Eisenstadt E, El-Dorry H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, Jaffe D, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CF, Miller JR, Montgomery P, Mori A, Nascimento AL, Naveira HF, Nusbaum C, O'Leary S, Orvis J, Pertea M, Quesneville H, Reidenbach KR, Rogers YH, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JM, Vanzee JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW. Genome sequence of *Aedes aegypti* a major arbovirus vector. Science. 2007; 316:1718–1723. [PubMed: 17510324]

Nimmo DD, Alphey L, Meredith JM, Eggleston P. High efficiency site-specific genetic engineering of the mosquito genome. Insect Mol. Biol. 2006; 15:129–136. [PubMed: 16640723]

Nolan T, Petris E, Muller HM, Cronin A, Cateruccia F, Crisanti A. Analysis of two novel midgut-specific promoters driving transgene expression in *Anopheles stephensi* mosquitoes. PLoS One. 2011; 6(2):e16471. [PubMed: 21326609]

Noriega FG, Edgar KA, Bechet R, Wells MA. Midgut exopeptidase activities in *Aedes aegypti* are induced by blood feeding. J. Insect Physiol. 2002; 48:205–212. [PubMed: 12770120]

Noriega FG, Pennington JE, Barillas-Mury C, Wang XY, Wells MA. Early trypsin, an *Aedes aegypti* female specific protease, is post-transcriptionally regulated by the blood meal. Insect Mol. Bio. 1996; 5:25–29. [PubMed: 8630532]

O'Kane CJ, Gehring WJ. Detection in situ of genomic regulatory elements in Drosophila. Proc. Natl. Acad. Sci. USA. 1987; 84:9123–9127. [PubMed: 2827169]

Ornitz DM, Moreadith RW, Leder P. Binary system for regulating transgene expression in mice: targeting int-2 gene expression with yeast GAL4/UAS control elements. Proc. Natl. Acad. Sci. USA. 1991; 88:698–702. [PubMed: 1846961]

Papathanos PA, Windbichler N, Menchinelli M, Burt A, Crisanti A. The *vasa* regulatory region mediates germline expression and maternal transmission of proteins in the malaria mosquito *Anopheles gambiae*: a versatile tool for genetic strategies. BMC Mol Biol. 2009; 10:65. [PubMed: 19573226]

Raikhel, AS.; Brown, MR.; Belles, X. Endocrine Control of Reproductive Processes. In: Gilbert, LI.; Iatrou, K.; Gill, S., editors. Comprehensive Molecular Insect Science. Vol. 3, Endocrinology. Elsevier Press; 2005. p. 433-491.

Riehle MA, Fan Y, Cao C, Brown MR. Molecular characterization of insulin-like peptides in the yellow fever mosquito *Aedes aegypti*: expression, cellular localization, and phylogeny. Peptides. 2006; 27:2547–2560. [PubMed: 16934367]

Roy SG, Hansen IA, Raikhel AS. Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol. 2007; 37:1317–1326. [PubMed: 17967350]

Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr. Opin. Cell. Biol. 2005; 17:596–603. [PubMed: 16226444]

Scheer N, Campos-Ortega JA. Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. Mech. Dev. 1999; 80:153–158. [PubMed: 10072782]

Schinko JB, Weber M, Viktorinova I, Kiupakis A, Averof M, Klinger M, Wimmer EA, Bucher G. Functionality of the GAL4/UAS system in Tribolium requires the use of endogenous core promoters. BMC Dev. Biol. 2010; 10:53–64. [PubMed: 20482875]

Wallrath LL, Elgin SC. Position effect variegation in Drosophila is associated with an altered chromatin structure. Genes Dev. 1995; 9(10):1263–1277. [PubMed: 7758950]

Wen Z, Gulia M, Clark KD, Dhara A, Crim JW, Strand MR. Two insulin-like peptide family members from the mosquito *Aedes aegypti* exhibit differential biological and receptor binding activities. Mol. Cell Endocrinol. 2010; 328:47–55. [PubMed: 20643184]

We generated CP-Gal4 *Aedes aegypti* mosquitoes with a midgut-specific expression.

The CP-Gal4 drives expression in the female mosquito midgut after blood feeding.

Insulin and Target-of-Rapamycin regulate the carboxypeptidase *CP* gene.

RNA interference silencing of the insulin receptor has confirmed this finding.

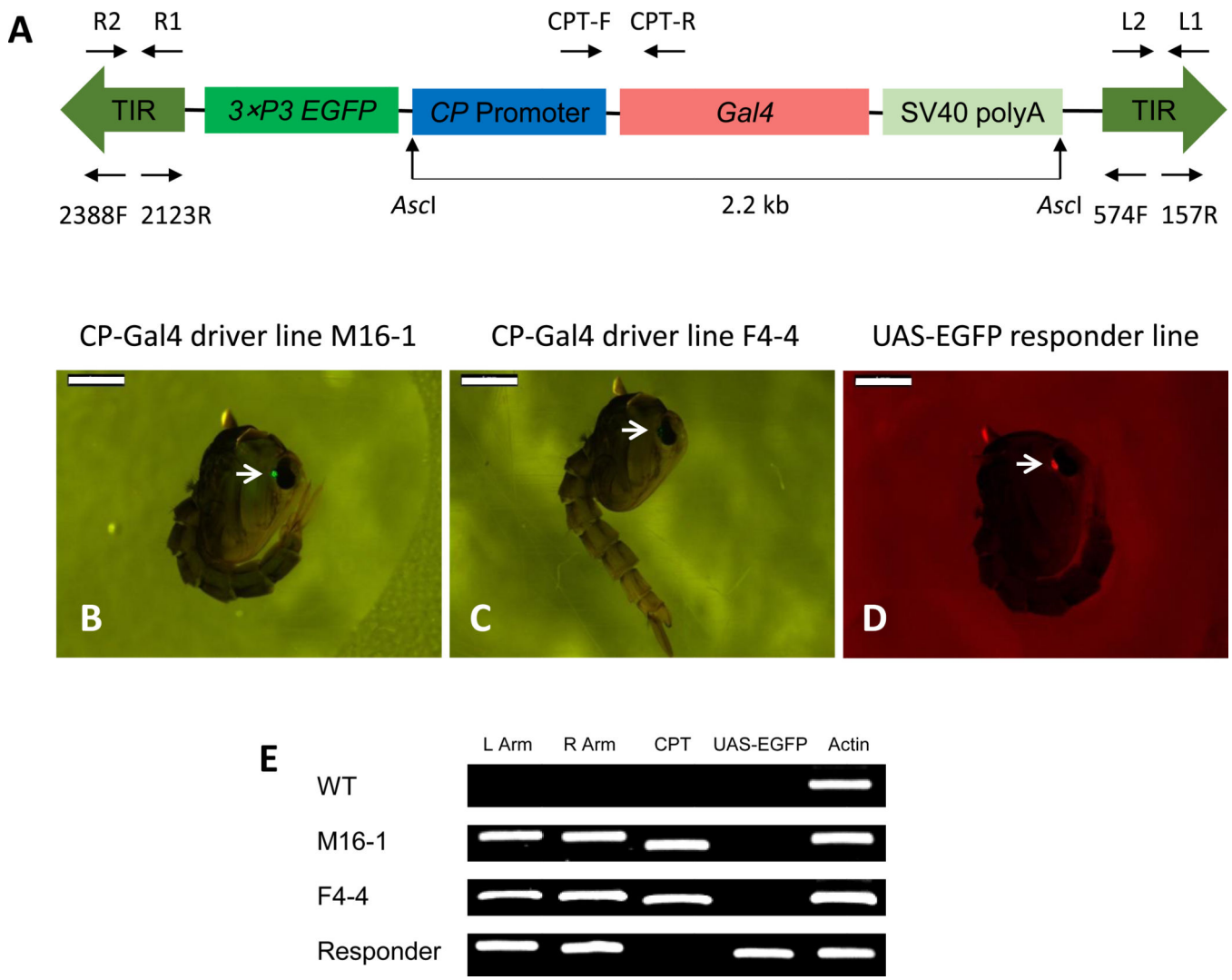

Figure 1.

Germline transformation and molecular characterization of *Ae. aegypti* CP-Gal4 lines. (A) Schematic representation of the CP-Gal4 cassette. The CP-Gal4 cassette consists of the *piggyBac* vector plasmid containing the 5' regulatory region of the CP gene placed upstream of the modified *Gal4* and the *EGFP* marker gene under the control of the eye-specific *3×P3* gene promoter. Arrows above and below the A diagram represent the transgene-specific primers used for genomic PCR, RT-PCR and inverse PCR. (B–D) Eye-specific expression of the selectable markers, EGFP and DsRed, in *Ae. aegypti* transgenic mosquito lines. Transgenic mosquitoes were selected based on the presence of EGFP marker in CP-Gal4 lines and DsRed marker in the UAS-EGFP line at the pupal stage (arrows). Screening was carried out using a Nikon SMZ800 fluorescence microscope with GFP-B and DsRed filters, respectively. Scale bar: 1mm. (E) Genomic PCR analysis demonstrating integration of CP-Gal4 and UAS-EGFP constructs into the *Ae. aegypti* genome. Wild type (WT), CP-Gal4 driver line M16-1 (M16-1), CP-Gal4 driver line F4-4 (F4-4) and UAS-EGFP responder line (Responder), were checked. Primers were specific to the left and right arms of the piggyBac

vector, left arm (L Arm), right arm (R Arm), the *CP-Gal4* transgene (CPT) and the *UAS-EGFP* transgene (UAS-EGFP). Primers for actin were used as control (Actin).

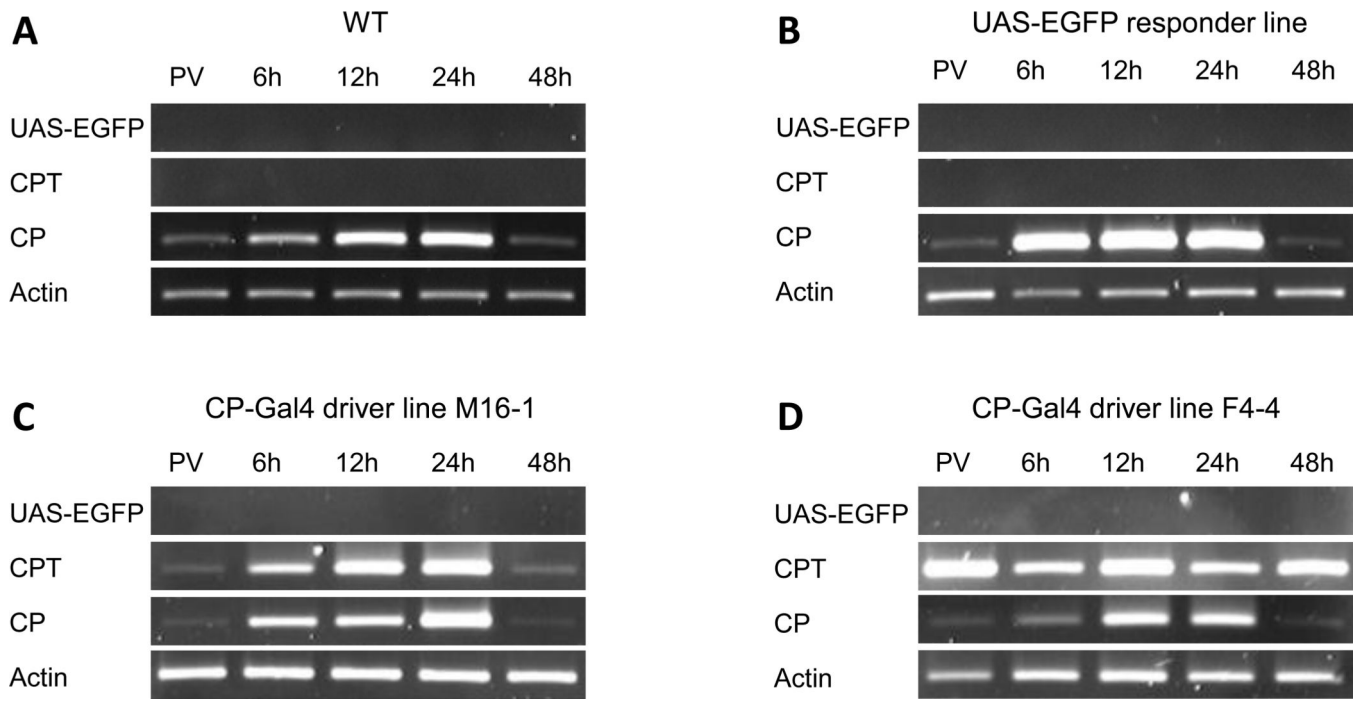

Figure 2.

RT-PCR analysis of the *CP-Gal4* transgene expression in *Ae. aegypti* females. (A) WT, (B) UAS-EGFP responder line, (C) M16-1 CP-Gal4 driver line, (D) F4-4 CP-Gal4 driver line. Primers were specific to the *UAS-EGFP* transgene (UAS-EGFP), *CP-Gal4* transgene (CPT) and endogenous *CP* gene (CP). Actin primers were used as control (Actin). RNA samples were extracted from isolated digestive systems of female mosquitoes at different time points: 72-h-old mosquitoes prior to blood feeding (PV), 6, 12, 24 and 48 hours PBM.

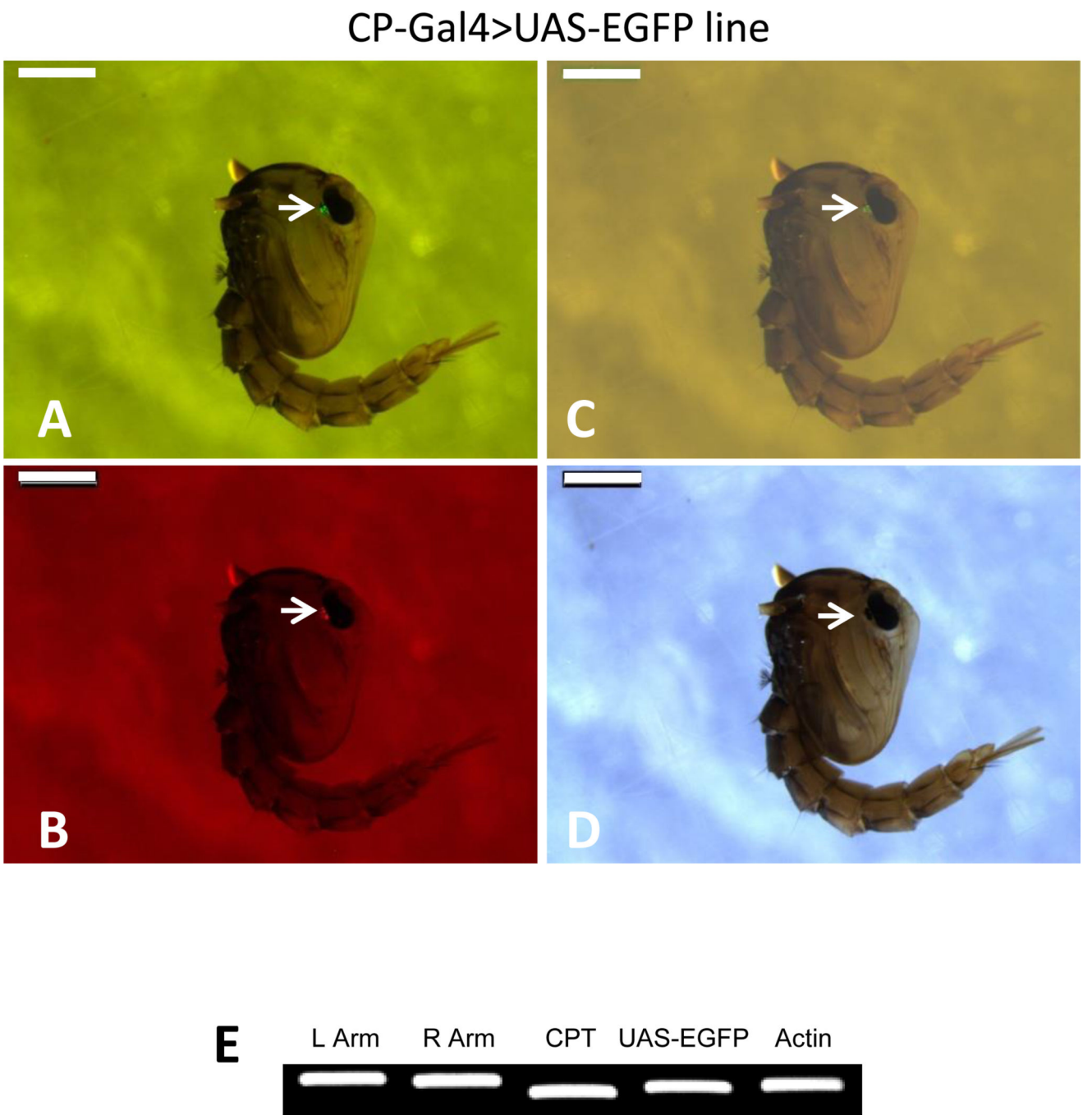

Figure 3.

Establishment of the *Ae. aegypti* CP-Gal4>UAS-EGFP hybrid line. (A–D). Pupae of hybrid mosquitoes were screened for the presence of eye-specific expression of the selectable markers, EGFP and DsRed. (A) GFP-B filter; (B) DsRed filter; (C) merged; (D) white light. Scale bar: 1mm. Nikon SMZ800 fluorescence microscope with GFP-B and DsRed filters was used for screening. (E) Genomic PCR analysis for verifying the integration of the CP-Gal4 driver and UAS-EGFP responder constructs in the *Ae. aegypti* CP-Gal4>UAS-EGFP hybrid line. Specific primers used for the analysis: L Arm and R Arm – left and right arms

of the piggyBac vector; CPT - *CP-Gal4* transgene; and UAS-EGFP - *UAS-EGFP* transgene. Actin probe was used as the loading control.

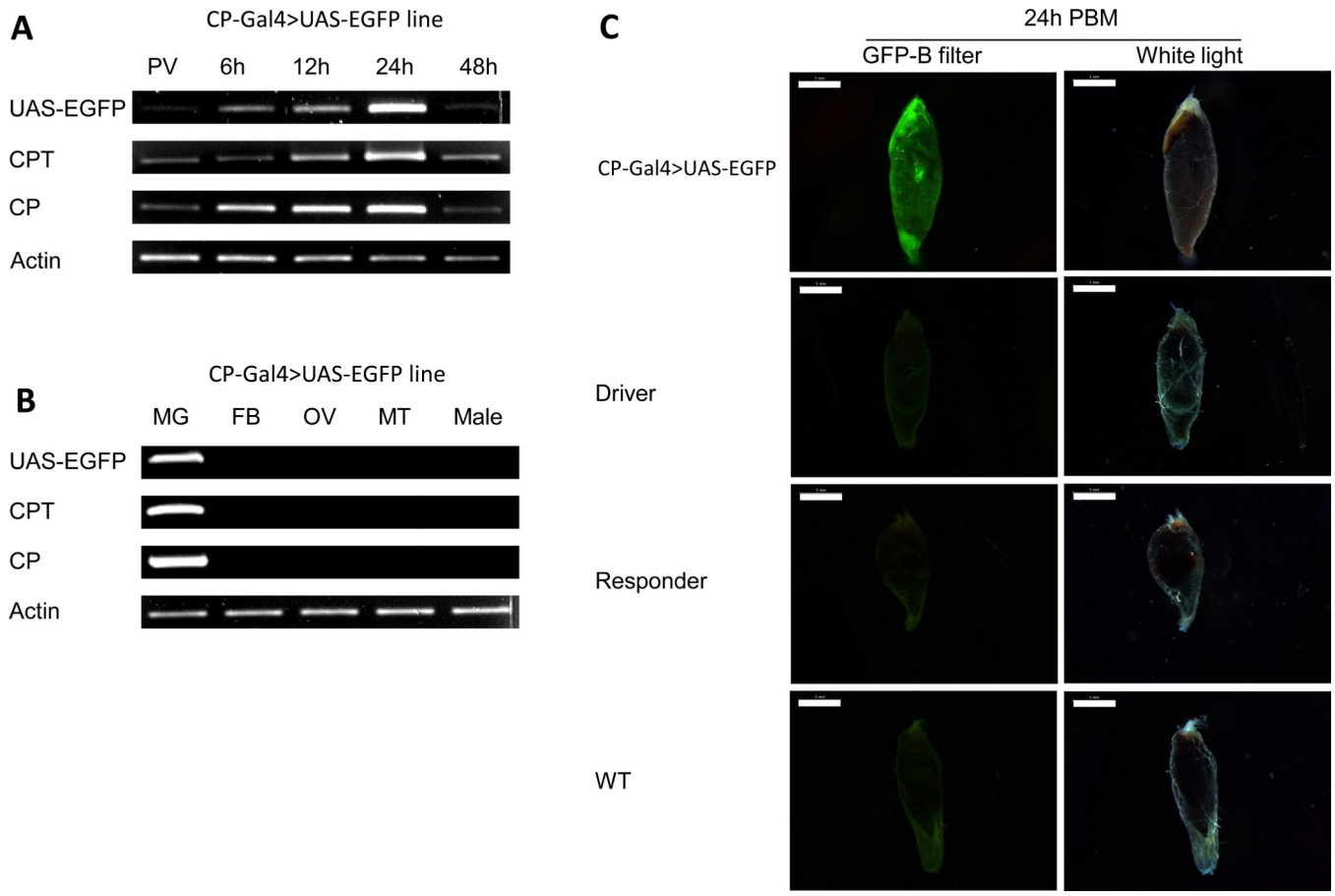

Figure 4.

Expression of the *CP-Gal4>UAS-EGFP* transgene in the *Ae. aegypti* hybrid line. (A) The time course of transcript abundance in midguts of hybrid transgenic female mosquito line using gene-specific primers. RNA samples were extracted from different time points: PV, 6, 12, 24 and 48 h PBM. (B) Tissue- and sex-specific expression of the *CP-Gal4>UAS-EGFP* transgene in the CP-Gal4>UAS-EGFP hybrid line mosquitoes. RNA samples were extracted from midgut (MG), fat bodies (FB), ovaries (OV), Malpighian tubules (MT) of females at 24 h PBM and adult hybrid males (M). Analyses in (A) and (B) were performed by means of RT-PCR using UAS-EGFP, CPT and CP probes. Actin was used as a loading control in (A) and (B). (C) EGFP expression in midguts from females of the CP-Gal4>UAS-EGFP line, CP-Gal4 driver line M16-1 (driver), UAS-EGFP responder line (responder), and wild type (WT). Midguts were dissected at 24 h PBM. Images were obtained using Leica M165FC fluorescent stereomicroscope using GFP-B filter or transparent filter (White light) and LAS V4.0 software. Scale bar: 1mm.

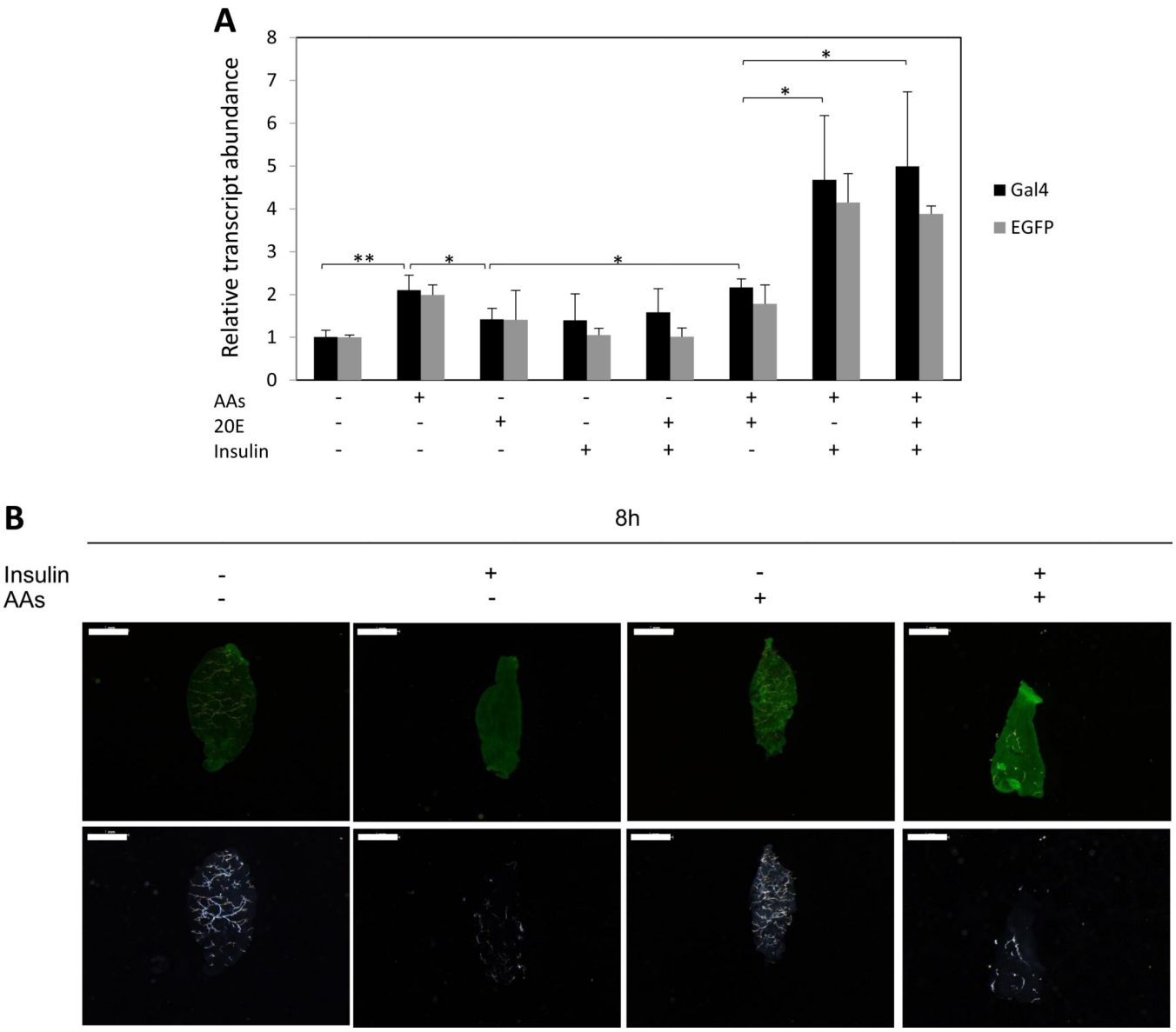

Figure 5.

Effects of AAs, insulin and 20E on expression of the *CP-Gal4>UAS-EGFP* transgene. (A) Midguts were isolated from non-blood fed CP-Gal4>UAS-EGFP female mosquitoes and incubated under different indicated conditions *in vitro* for 8 h. Levels of the *CP-Gal4>UAS-EGFP* transgene transcript were monitored using Gal4 and EGFP primers (Table S3) by means of qRT-PCR. Control transcript level was set at 1, while transcript levels for other treatments were expressed relative to the control. Values are the means of three replicates (± SEM). The experiment was repeated three times. * Indicates statistical significance <0.05. ** Indicates statistical significance <0.01. (B) Detection of EGFP protein in midguts isolated from non-blood fed CP-Gal4>UAS-EGFP female mosquitoes by means of fluorescent immunocytochemistry. The midguts were cultured for 8h under indicated conditions. Immunocytochemistry was performed using the anti- EGFP antibody, followed by incubation with an anti-rabbit fluorescent secondary antibody. Images were obtained

using Leica M165FC fluorescent stereomicroscope using GFP-B filter or transparent filter (White light) and LAS V4.0 software. Scale bar: 1mm.

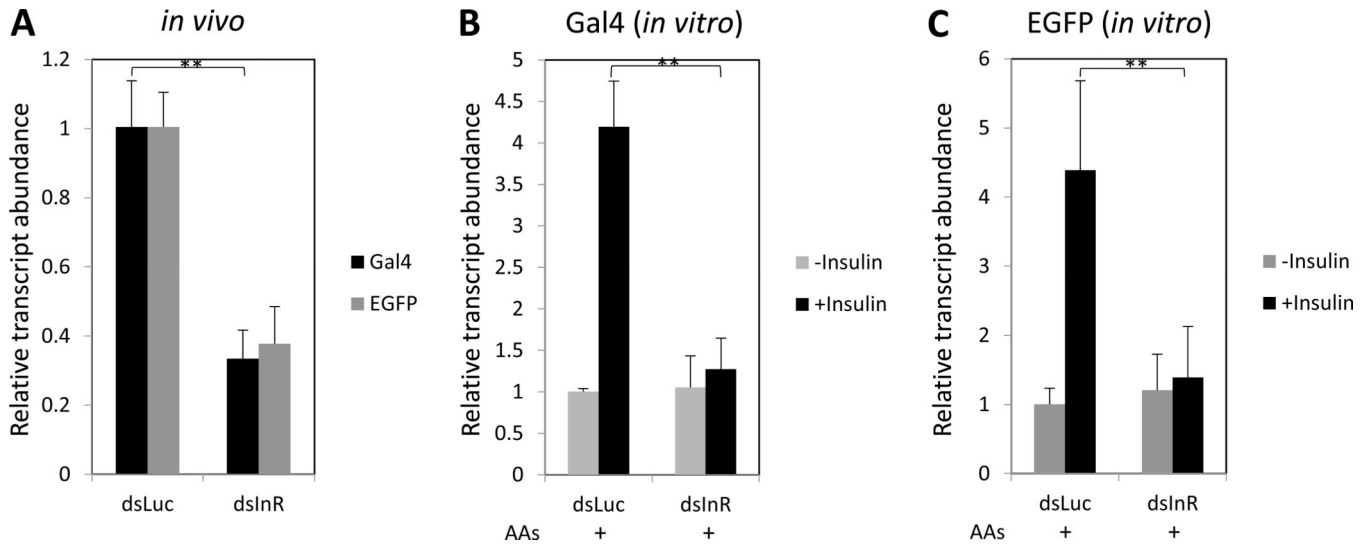

Figure 6.

Insulin signaling pathway controls expression of the *CP-Gal4>UAS-EGFP* transgene. (A) RNAi of Insulin Receptor (dsInR) *in vivo*. RNAi for Luciferase (dsLuc) served as a control. The CP-Gal4>UAS-EGFP females were injected with dsRNAs at 1 day post-eclosion, blood fed and examined at 24 h PBM. Transcript abundance was examined using Gal4 and EGFP primers by means of qRT-PCR. Control dsLuc transcript level was standardized to 1, while transcript levels for other RNAi treatments were expressed relative to the control. (B–C). Activation of the *CP-Gal4>UAS-EGFP* transgene expression by insulin *in vitro* was prevented by dsInR. The CP-Gal4>UAS-EGFP females were injected with dsRNAs at 1-day post-eclosion, and an *in vitro* assay was performed as in Figure 5. Midguts were isolated from non-blood fed CP-Gal4>UAS-EGFP females and incubated in the AA-containing culture medium. Expression of the *CP-Gal4>UAS-EGFP* transgene was monitored by means of qRT-PCR using probes for Gal4 (B) and EGFP (C) (primer sequences in Table S3).

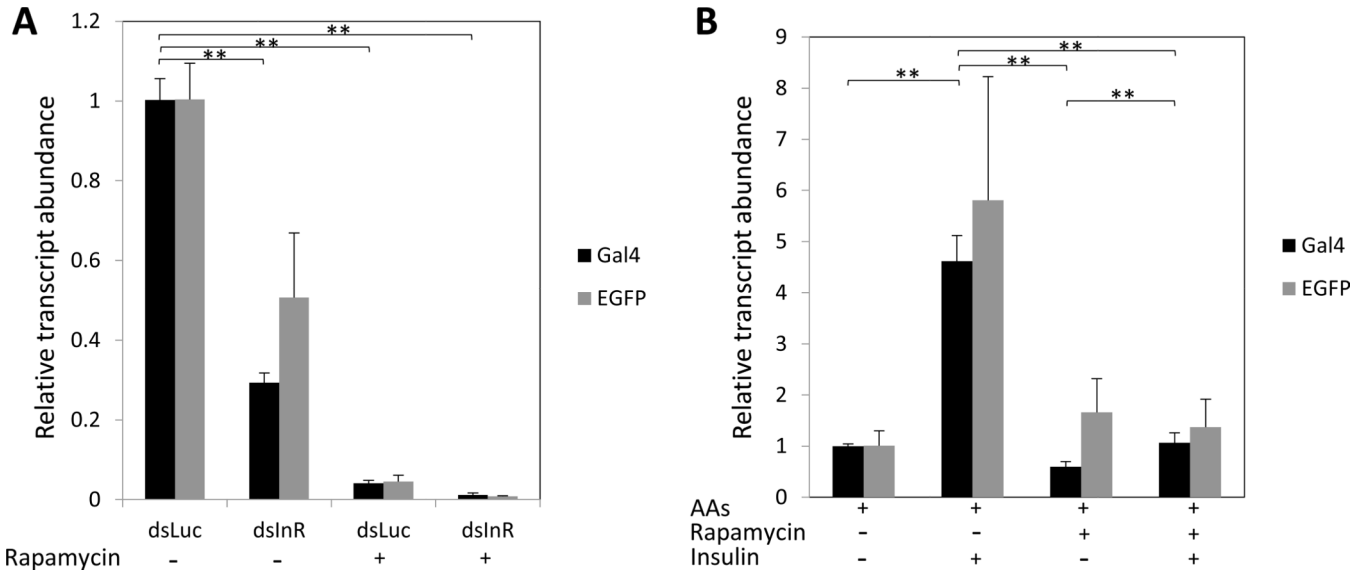

Figure 7.

Effect of TOR inhibitor rapamycin on the expression of the *CP-Gal4>UAS-EGFP* transgene. (A). *In vitro* application of AAs and insulin activated *CP-Gal4>UAS-EGFP* expression, and rapamycin was capable of blocking this activation. The transcript level was set at 1 for the "no treatment" sample (control), and transcript levels for the other treatments were expressed relative to the control. (B). qRT-PCR analysis of *CP-Gal4>UAS-EGFP* transgene expression in midguts at 24 h PBM from CP-Gal4>UAS-EGFP hybrid females after different treatments. 20 pmol of rapamycin was injected in each experiment. Transcript levels were standardized to 1 for the dsLuc sample (control), and transcript levels for the other treatment were expressed relative to the control.

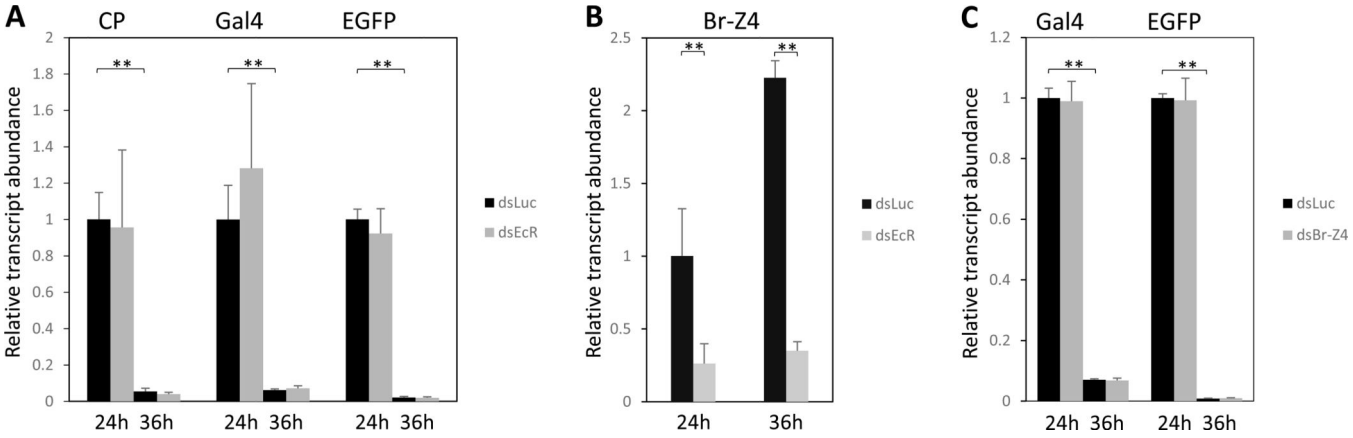

Figure 8.

RNAi of the *EcR* (dsEcR) and *Br-Z4* (dsBr-Z4) gene in the CP-Gal4>UAS-EGFP female mosquitoes. (A–C). The procedure was performed as in Figure 6A. RNAi for Luciferase (dsLuc) served as a control. The effect of the *EcR* and *Br-Z4* knockdown on the *CP-Gal4>UAS-EGFP* transgene expression was monitored by means of qRT-PCR using Gal4 and EGFP primers. The transcript levels of the endogenous *CP* gene (A) and *Br-Z4* gene (B) after dsEcR treatment were also examined. Times at which examinations were done are marked by 24h and 36h PMB.