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

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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 (TOR) kinase. 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-specific genes that are involved in blood digestion and interaction with pathogens in *Ae. aegypti* mosquitoes.

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

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 reaction; 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.

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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 and Campos-Ortega, 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 (2011) established the first binary Gal4/UAS system for the female-, blood-meal-induced- and fat-body-specific expression in the mosquito *Ae. aegypti*. Lynd and Lycett (2012) have reported the midgut-specific Gal4/UAS system in the malaria vector *An. gambiae*. 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–*Bam*HI site of the pSLfa1180fa shuttle vector (Horn and Wimmer, 2000) to generate pSL-CP. Then, a 0.8-kb *Bam*HI–*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 (0.35 mg/ml) and pAehsp-pBac helper (0.25 mg/ml) plasmids were re-suspended in 0.1 mM phosphate buffer (pH 6.8, containing 5 mM 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). G_1 progeny was 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, 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 ddH_2O. 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