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ORIGINAL ARTICLE

Expression of Foreign Proteins by Antimicrobial Peptide Gene Promoters in Mosquitoes

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Background: The mosquito *Aedes aegypti* is a major vector for transmission of viruses causing dengue fever, yellow fever, Zika, and chikungunya infection. Functional analysis of mosquito genes and individual viral genes can be a powerful approach to study vector–virus interactions but is often hampered by a lack of suitable promoters to drive exogenous viral gene expression in mosquito cells *in vitro* and *in vivo*. **Object:** To search for potential promoter candidates that can be used to express foreign genes and particularly viral proteins in a mosquito model system. **Materials and Methods:** we characterized the ability of the promoters of three *Ae. aegypti* antimicrobial peptide (AMP) genes to drive the expression of marker proteins (luciferase, GFP, the NS3 protein of two flaviviruses, and rabies virus glycoprotein) in mosquito cells and adult female mosquitoes, and in other insect cells as well. **Results:** The promoters of the defensin A4 and cecropin B1 genes produced robust expression of luciferase and GFP in the *Ae. aegypti* cell line CCL125, *Aedes albopictus* cell line C6/36, *Drosophila melanogaster* cell line S2, and *Spodoptera frugiperda* cell line Sf21. These AMP gene promoters also had the ability to drive NS3 and GFP expression in adult tissues of *Ae. aegypti*, *Ae. albopictus*, and *Culex tritaeniorhynchus in vivo*, which may suggest evolutionary conservation of AMP gene promoter activity across mosquito lineages. **Conclusions:** These promoters could provide a valuable tool for ectopically expressing viral genes and studying their interactions with the mosquito vectors.

Key words: Expression promoter, antimicrobial peptide gene, mosquito

INTRODUCTION

Aedes aegypti is the most important vector for transmission of viruses that cause dengue fever, yellow fever, chikungunya fever, and Zika fever worldwide. Currently, the most effective way to control mosquito-transmitted diseases is to reduce mosquito populations by habitat reduction and insecticide application, but such measures are labor intensive and can have unwanted impacts on the environment. The lack of effective vaccines (except for yellow fever) and the emergence of insecticide-resistant mosquitoes have urged the development of other control strategies, such as genetic approaches to generate mosquitoes with pathogen-resistant phenotypes.[1-4] Production of pathogen-resistant or immunocompetent transgenic mosquitoes requires gene promoters that can drive expression of foreign genes or siRNA in an appropriate manner.

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Several promoter fragments from genes of *Ae. aegypti* able to drive the expression of transgenes in transformed mosquitoes have been characterized, including the carboxypeptidase A promoter to drive inverted RNA of dengue virus 2 (DenV2) in midgut;[2] the vitellogenin promoter to strongly drive the defensin coding region in fat body and hemolymph following a blood meal[3] or to drive Rel2 in fat body, resulting in expression of defensins and cecropins after a blood meal;[1] the nanos promoter to express exogenous MosI transposase[5] in the ovary; the actin-4 promoter to induce a repressible female-specific flightless phenotype;[6] the beta2 tubulin promoter to express the DsRed gene in testis;[7] the Hsp70 promoter to drive expression of luciferase in adult mosquito after heat shock;[8] the Ub and polyubiquitin promoters to drive luciferase and GPF expression

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in culture and adult mosquitoes;[9] the Maltase-like I and apyrase gene promoters to drive constitutive expression of luciferase in *Aedes albopictus* C6/36, *Drosophila* Schneider Line 2 cells, and transformed mosquitoes;[10] the salivary protein 30 k promoter to drive an inverted repeat RNA with sequences derived from the premembrane gene of dengue 2 virus, resulting in reduced viral infection;[4] and three regulatory regions of the glutamine synthetase gene that differentially regulate its expression.[11] Of these promoters, some are tissue dependent,[2-5,7,12-14] some are stage dependent,[6,9,10] and some are feeding or temperature dependent.[1-3,8,15,16] The use of mosquito gene promoters for tissue- or stage-specific manipulation of the mosquito immune defense pathway is a powerful approach for understanding the gene function in the context of host–pathogen interactions as well as for counteracting mosquito-borne diseases.

In the present study, we analyzed three promoters (defensin A1, defensin A4, and cecropin B1) of antimicrobial peptide (AMP) genes and of one Hsp70 gene from *Ae. aegypti* for their ability to drive high constitutive expression of exogenous proteins (GFP and NS3 proteins of two flaviviruses) in the mosquito cell lines C6/36 and CCL125, *Drosophila* S2 cells, and *Spodoptera frugiperda* (moth) Sf21 cells. The A4 and B1 promoters functioned in adult female mosquitoes *in vivo*, expressing NS3 and GFP in tissues of *Ae. aegypti*, *Ae. albopictus*, and *Cx. tritaeniorhynchus*. The glycosylated G protein gene of rabies virus was also expressed in mosquito cells under the control of B1 promoter. The results revealed that the promoter activities of these AMP genes from *Ae. aegypti* are conserved evolutionarily in the dipteran lineage and could be a valuable tool for expressing viral proteins and assaying their interactions with host proteins of mosquito vectors.

MATERIALS AND METHODS

Mosquito rearing

The *Ae. aegypti* Kaohsiung strain, *Ae. albopictus* Chung-Ho strain, and *Cx. tritaeniorhynchus* Peitou strain were reared at 27ºC and 80% humidity, as described previously.[17] Adult female mosquitoes used for intrathoracic injection were aged 1–5 days after emergence from pupal cases.[18,19]

Cell lines

Mosquito cell lines – CCL125 (ATCC, *Ae. aegypti*) and C6/36 (*Ae. albopictus*) were cultured in RPMI 1640 medium (GIBCO, Invitrogen, CA) with 10% fetal bovine serum, 50 units/ml penicillin G, 50 mg/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES in a humidified atmosphere of 5% CO_2 at 28ºC. The S2 cell line (*Drosophila melanogaster*) was cultured in Schneider's insect medium (USBiological, MA) with 10% fetal bovine serum in 5% CO_2 28ºC. The Sf21 cell line (*S. frugiperda*) was cultured in TC100 medium (USBiological, MA) with 10% fetal bovine serum in 5% CO_2 at 28ºC. Rodent BHK21 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum.

DNA constructs

The promoters of the defensin A1, defensin A4, cecropin B1, and heat shock protein (Hsp) genes from *Ae. aegypti* were polymerase chain reaction (PCR) amplified using adult mosquito genomic DNA as template and the following primers: A1-forward ACACGCTAGAACACGTAAG, A1-reverse GATAATCGACGAGCTCTGCG; A4-forward AATTCGGTGACCCACTAGATC, A4-reverse GTG CTAATCAAACAGCTGAG; B1-forward GCC TACACTCAAATGTTCATCAATGG, B1-reverse TC AAGCTTTCACTGGAATAGGTGA; Hsp-forward TCC AGTCTTTTTGAAGTCGCGAA, Hsp-reverse CTTTAA TTAGTGTTGTTTTGACGAGA. The sizes of the amplified A1, A4, B1, and Hsp promoters were 1063nt, 1239nt, 1868nt, and 675nt, respectively, and all were cloned into pGL3 basic vector (Promega, WI, USA) between the *Nhe*I and *Bgl*II restriction enzyme sites and tested for luciferase expression. The luciferase gene in the pGL3 basic-A1, pGL3 basic-A4, and pGL3 basic-B1 constructs were replaced with the GFP gene or the NS3 gene of Japanese encephalitis virus (JEV) or DenV2 between the *Nco*I and *Xba*I sites for GFP or NS3 protein expression. The GenBank accession numbers are HQ256583 and HQ256580 for NS3 of JEV and DenV2, respectively. pCMV-GFP (Invitrogen, CA, USA) was used as a GFP control plasmid. The plasmids – pGL3-A1, pGL3-A4, pGL3-B1, pGL3-Hsp, pGL3-A1-GFP, pGL3-A4-GFP, pGL3-B1-GFP, pGL3-A4-JENS3, pGL3-B1-JENS3, pGL3-A4-D2NS3, pGL3-B1-D2NS3, and pCMV-GFP were sequenced to confirm the insertions and prepared by QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany).

The rabies virus G protein gene plasmid (pGL3-B1-G) was constructed using inactivated VERORAB vaccine (Sanofi, Lyon, France) to obtain RNA template and RT-PCR methods (using primers forward-GGGAAGTTCCCCATTTACACGAT, reverse-CTGTTTATGCACATCGGGGAG) to amply the G-specific coding region, which was used to replace the luciferase gene between the *Nco*I and *Xba*I restriction enzyme sites downstream of the B1 promoter. The G protein gene of pGL3-B1-G was sequenced and found to be identical to the G gene of the rabies virus PM strain (GenBank accession number AJ871962), with 3–4 potential N-glycosylation sites within the encoded protein.

Luciferase assays

C6/36, CCL125, S2, and Sf21 cells were cultured in 12-well plates at about 1×10^5 cells per well. The plasmids – pGL3-A1,

pGL3-A4, pGL3-B1, and pGL3-Hsp (0.2 mg/well) were transfected into C6/36 and CCL125 cells using Fugene HD transfection reagent (Roche, Indiana) and into S2 and Sf21 cells using Cellfectin II reagent (Invitrogen, CA, USA) according to the manufacturers' instructions. The cells were lysed with passive lysis buffer (Promega, CA, USA), and luciferase activity was assayed (BioThema, Sweden) in a microplate scintillation format and luminescence counter (Packard, PerkinElmer, CT, USA) 48-h posttransfection. The luciferase activities were normalized with the protein concentration in each well. *In vivo* luciferase assays were performed by mixing the plasmids with TurboFect™ *in vivo* reagent (Fermentas, Canada), followed by intrathoracic microinjection of 98 nl (~50 ng of plasmid DNA) into adult female mosquitoes;[18,19] homogenates of individual mosquitoes were assayed for luciferase activity 5-day postinjection.

Microscopy and fluorescence analysis

Expression of GFP from the constructs – pGL3-A1-GFP, pGL3-A4-GFP, and pGL3-B1-GFP in C6/36, CCL125, and S2 cells was detected by fluorescence microscopy (IX70, Olympus), and images were captured by a SPOT RT3™ camera (SPOT™ Imaging Solutions, Sterling Heights, MI). Expression of GFP in fat body of female adult mosquitoes was detected 5 days after intrathoracic microinjection[19] of 98 nl (~50 ng plasmid DNA) of pGL3-A4-GFP or pGL3-B1-GFP by microdissecting fat body onto a slide and examining the tissue by fluorescence microscopy (Zeiss Axio Scope A1 fluorescent microscope; Carl Zeiss, Germany); images were captured with Canon EOS 450D digital system (Canon, Japan).

Antibodies and Western blotting

Polyclonal antibody against GFP was purchased from Gene Tex (San Antonio, TX, USA). Monoclonal antibody against alpha-tubulin was purchased from Sigma-Aldrich Biotechnology (St. Louis, MO, USA). Monoclonal anti-rabies G antibody was from Applied Biological Materials (Richmond, BC, Canada). Rabbit polyclonal antisera specifically recognizing aegSTAT and albSTAT were generated using a His-tagged aegSTAT or albSTAT C-terminal fusion protein as antigen to immunize New Zealand White rabbits.[17] Monoclonal antibodies against NS3 of JEV and DenV2 were both from the Institute of Preventive Medicine, NDMC (Taiwan, ROC). The C6/36, CCL125, and S2 cells transfected with the indicated plasmids for 48 h were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM ethylenediaminetetraacetic acid) containing 2 mM phenylmethylsulfonyl fluoride and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to a nitrocellulose membrane and blocked with 5% skim milk in PBS + 0.1% Tween 20 (PBST). The membrane was incubated with the indicated primary antibody and secondary horseradish peroxidase antibody and washed three times with PBST after antibody incubation, then visualized by using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE, Healthcare, UK) according to the manufacturer's instructions.

Immunohistochemistry

Adult injected with 50 ng expression vectors (pGL3-A4 or B4-JENS3) for 5 days, mosquitoes were fixed in 10% buffered formalin, paraffin-embedded, and 5-microm cut slices were dewaxed in xylene, rehydrated through graded alcohol, and subjected to an endogenous peroxide blocking. The sections were washed in water, processed for antigen retrieval, and placed in citrate buffer. Nonreactive staining was blocked by treating the sections with 1% goat normal serum in Tris-buffered saline for 20 min. Tissue sections were incubated with anti-NS3 antibody of JEV for 1 h, washed with Tris-buffered saline three times, and then, the bound antibodies were detected using an avidin-biotin-peroxidase complex (Universal Elite ABC kit, Vectastain, CA, USA) for 30 min. Diaminobenzidine tetrahydrochloride solution (Kit HK153-5, Biogenex, CA, USA) was used as a chromogen. Images were detected with a Zeiss Axio Scope A1 microscope (Carl Zeiss, Germany) and captured using a Canon EOS 450D digital system (Canon, Japan).

Data analysis

Figures 1a, b, and Figure 2b were constructed using GraphPad Prism software. Statistical analyses for significant differences were carried out with an unpaired *t*-test using the same software (GraphPad software, San Diego, CA).

RESULTS

Construction of protein expression vectors containing promoters of antimicrobial peptide genes from *Aedes aegypti*

Promoters of the defensin A1, defensin A4, cecropin B1, and heat shock protein genes of the *Ae. aegypti* Kaohsiung strain were PCR amplified from adult female genomic DNA and cloned into pGL3 basic vector to create pGL3-A1, pGL3-A4, pGL3-B1, and pGL3-Hsp. The GenBank accession numbers of these promoters are HQ285957 to HQ285960. The luciferase gene in these modified pGL3 basic constructs was further replaced by the NS3 gene of JEV or DenV2, the G gene of rabies virus, or the GFP gene to give the following expression vectors: pGL3-A1-GFP, pGL3-A4-GFP, pGL3-B1-GFP, pGL3-A4-JENS3, pGL3-B1-JENS3, pGL3-A4-D2NS3,

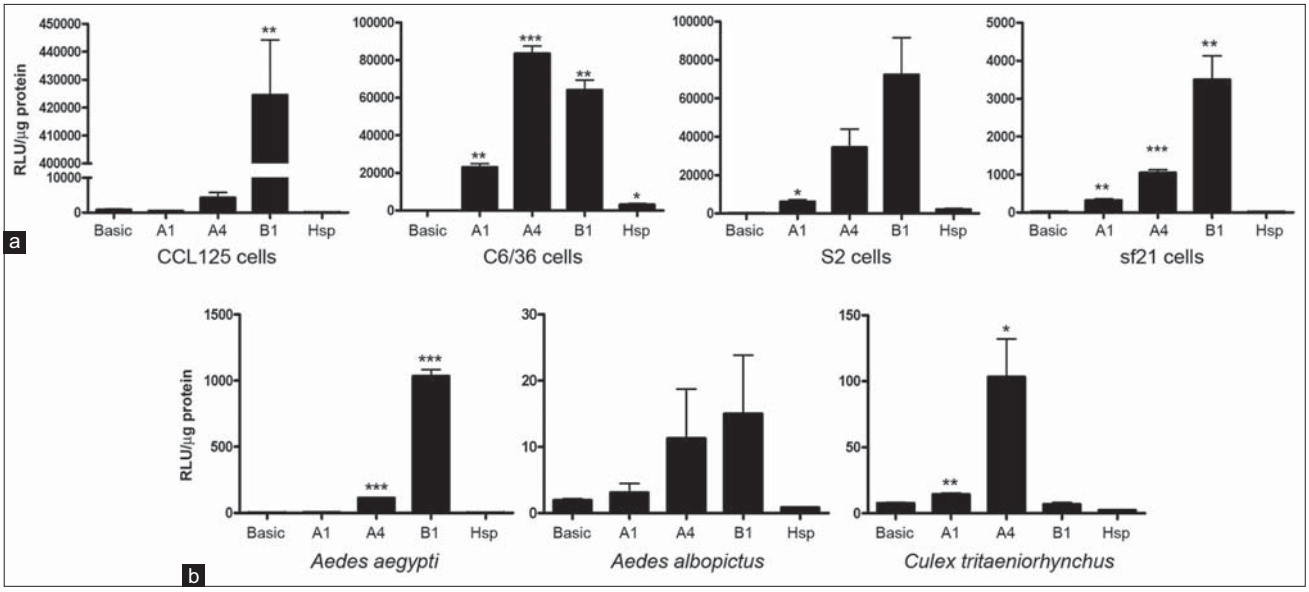

Figure 1: (a) Luciferase activity assays of four mosquito promoters in insect cells. Mosquito cell lines – CCL125 and C6/36, *Drosophila* cell line S2, and moth cell line Sf21 were transiently transfected with luciferase reporter constructs each containing one of four mosquito promoters (A1, A4, B1, Hsp) and a pGL3-Basic vector as a control. Luciferase activity was measured 48-h posttransfection. Relative luciferase activity was normalized to cellular protein concentration. Bar values represent the means of three individual experiments ± standard error of the mean. Significant differences between each promoter reporter and the pGL3-Basic vector were determined by unpaired *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (b) *In vivo* expressed luciferase activities controlled by four mosquito promoters in three mosquito species. Adult females of three different mosquito species, *Aedes aegypti*, *Aedes albopictus*, and *Culex tritaeniorhynchus,* were intrathoracically injected with luciferase reporters containing four different promoters (A1, A4, B1, Hsp) and control pGL3-Basic vector. Luciferase activity was measured 5-day postinjection. Relative luciferase activity was normalized to each individual mosquito protein concentration. Bars represent the means of three different adult mosquito experiments ± standard error of the mean. Significant differences between each reporter and the pGL3-Basic vector (Basic) were determined by unpaired *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

pGL-B1-D2NS3, and pGL3-B1-G. A diagram of the plasmid constructs is shown in Figure 3.

Mosquito promoters drive expression of luciferase reporter in several insect cell lines and adult mosquitoes

The four luciferase reporters – pGL3-A1, pGL3-A4, pGL3-B1, and pGL3-Hsp (containing mosquito promoters A1, A4, B1, and Hsp) – showed varying levels of luciferase activity in the two mosquito cell lines – CCL125 (*Ae. aegypti*) and C6/36 (*Ae. albopictus*) and the two insect (nonmosquito) cell lines – S2 (fruit fly) and Sf21 (lepidopteran), with the B1 and A4 promoters driving higher luciferase expression than the A1 and Hsp promoters in all four cell lines [Figure 1a]. In CCL125, the luciferase activity driven by the B1 promoter was about 80-fold higher than that driven by the A4 promoter; by contrast, in the heterologous C6/36 cell line, the B1 promoter was less active than the A4 promoter. The same pattern of luciferase activity as observed in the CCL125 cell line was also observed in the S2 and Sf21 cell lines, with activity highest for the B1 promoter, moderate for the A4 promoter, and lowest for A1 and Hsp promoters [Figure 1a]. The above results demonstrated that promoters from *Ae. aegypti* can drive luciferase expression both in mosquito cell lines and in other insect cell lines, but generally appear to be more efficient in mosquito cells.

To further test that these promoters could drive luciferase activity in adult mosquitoes, the luciferase-containing reporters were intrathoracically injected into adult female mosquitoes of *Ae. aegypti*, *Ae. albopictus*, and *Cx. tritaeniorhynchus*, and then homogenates of individual adult mosquitoes were assayed for luciferase activity 5-day postinjection. Figure 1b shows that luciferase expression was more vigorous in *Ae. aegypti* adult mosquito than in both *Ae. albopictus* and *CX. tritaeniorhynchus* adults, and the B1 promoter was more active than A4 and A1 in *Aedes* mosquitoes whereas the A4 promoter was more active than B1 and A1 in *Culex* mosquitoes. These results demonstrated that mosquito promoters not only can drive luciferase expression in cell lines but also in adult mosquitoes *in vivo*.

Mosquito promoters drive expression of GFP in insect cell lines and glycosylated G protein of rabies virus in mosquito cells

To test whether these mosquito gene promoters could drive expression of other exogenous proteins, GFP gene was substituted for the luciferase gene in pGL3-A1, pGL3-A4,

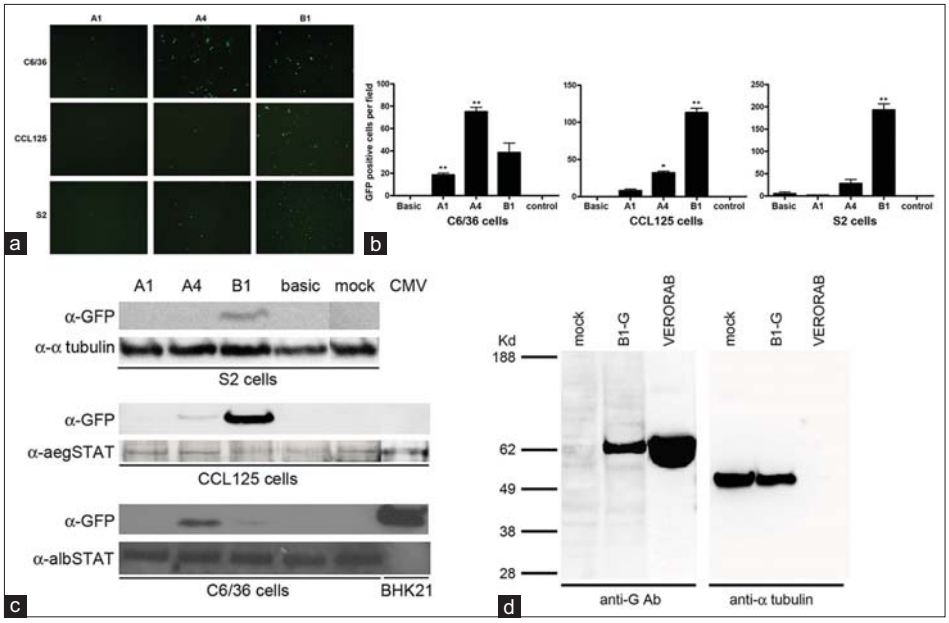

Figure 2: GFP, driven by three different mosquito promoters, was expressed in three insect cell lines. (a) GFP reporter plasmids – pGL3-A1-GFP, pGL3-A4-GFP, and pGL3-B1-GFP were used to transiently transfect C6/36, CCL125, and S2 cells, and GFP was detected by fluorescent microscopy. Each image was magnified 100×. Mock and pGL3-Basic control assays are not shown. (b) Bars represent the mean number of GFP-positive cells per visual field from three different experiments (*$P < 0.05$, **$P < 0.01$). (c) GFP expressed in insect cells was detected by Western blotting. Mosquito cell lines – CCL125 and C6/36 and *Drosophila* cell line – S2 were transiently transfected with pGL3-A1-GFP, pGL3-A4-GFP, pGL3-B1-GFP, and pGL3-Basic plasmids or mock transfected, cells were lysed 48 h after transfection, and the lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane and detected by anti-GFP antibody. Mammalian cell line BHK21 was transfected with pCMV-GFP plasmid as a positive control. CCL125 cells transfected with pCMV-GFP plasmid showed that GFP was not expressed from the CMV promoter. (d) Glycosylated G protein of rabies virus was also expressed in mosquito cell line CCL125 under the control of the B1 promoter. This G protein construct (pGL3-B1-G) was transfected into the CCL125 cell line for 48 h, the cells lysed for Western blotting, and the blot probed with anti-G monoclonal antibody and reprobed with antialpha tubulin antibody. Inactivated rabies vaccine VERORAB (3 ml) in lane 3 served as a positive control

and pGL3-B1 to create pGL3-A1-GFP, pGL3-A4-GFP, and pGL3-B1-GFP [Figure 3]. C6/36, CCL125, and S2 cells were transfected with these GFP reporters and examined by fluorescence microscopy 48-h posttransfection. GFP was expressed in all three insect cell lines, with B1 promoter being the strongest in CCL125 and S2 cells and the A4 promoter more active in C6/36 cells [Figure 2a]. For quantitative analysis of the GFP-expressing cells, three independent transfection assays were carried out in each of C6/36, CCL125, and S2 cells, and the numbers of GFP-positive cells were counted in 5 visual fields per experiment to compare the GFP fluorescence among the A1, A4, and B1 promoters [Figure 2b]. The results showed that the B1 promoter was more active in CCL125 and S2 cells, whereas A4 was more active in C6/36 cells, consistent with the results of the luciferase activities in Figure 1a. GFP expression was also detected in lysates of these cells by Western blotting [Figure 2c]. The results revealed GFP protein to be more abundant in CCL125 and S2 cells transfected with the B1 promoter construct and in C6/36 cells transfected with the A4 promoter construct, consistent with the results of the GFP experiments in Figures 1a and 2a, b. As a negative control, the CMV promoter could drive GFP expression in mammalian BHK21 cells as expected, but not in mosquito CCL125 cells [Figure 2c]. To test whether a glycosylated foreign protein could be produced from these mosquito promoters in mosquito cells, the coding region of the G protein gene of rabies virus was inserted downstream of the B1 promoter and the construct was transfected into CCL125 cells; the results showed that glycosylated G protein could be expressed under these conditions [Figure 2d].

Antimicrobial peptide promoters drive GFP expression in adult mosquitoes *in vivo*

We further assayed the expression of GFP under the control of the A4 and B1 promoters in *Ae. aegypti* adult females by intrathoracic injection of pGL3-A4-GFP and pGL3-B1-GFP plasmid (~50 ng DNA per adult mosquito). Microdissected fat body showed GFP fluorescence in both the A4 and B1 promoter transfection experiments, as visualized by fluorescence microscopy [Figure 4]. This result showed that promoters of *Ae. aegypti* can drive expression of foreign GFP protein in fat body of adult mosquitoes *in vivo*.

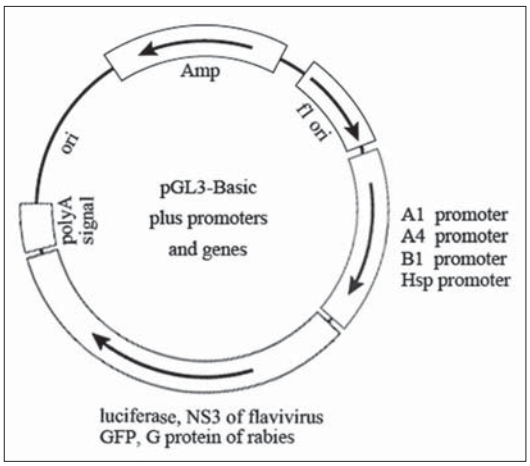

Figure 3: Diagram of plasmids showing mosquito promoters (A1, A4, B1, Hsp), exogenous protein genes (luciferase, GFP, G protein of rabies virus, NS3 gene of flavivirus), and the pGL3-Basic vector backbone

Antimicrobial peptide promoters drive expression of flavivirus NS3 proteins in mosquito cell lines and adult mosquitoes

The above experiments revealed that mosquito gene promoters are able to drive foreign luciferase and GFP protein expression in insect cells *in vitro* and *in vivo*. We next replaced the GFP gene with the NS3 gene of JEV or DenV2 to test whether mosquito AMP gene promoters could also drive the expression of this viral protein in mosquito cells. The NS3 protein of JEV was detected in C6/36 cells after transfection with pGL3-A4-JENS3 and pGL3-B1-JENS3 plasmids, and the NS3 of DenV2 was expressed in CCL125 cells transfected with pGL3-A4-D2NS3 and pGL3-B1-D2NS3 [Figure 5a]. These data revealed that the promoters of these AMP genes from mosquito could be used to drive individual viral gene expression in mosquito cell lines without whole virus infection. We further tested whether these promoters could drive JEV NS3 gene expression in adult mosquito by intrathoracic injection of *Ae. albopictus* [Figure 5b, upper panels, middle, and right] and *Cx. tritaeniorhynchus* [Figure 5b, lower panel, middle, and right] females with pGL3-A4-JENS3 or pGL3-B1-JENS3 plasmid (~50 ng DNA per adult mosquito) for 5 days. The injected mosquitoes were formalin fixed and paraffin embedded, and sliced sections were dewaxed in xylene, rehydrated through graded alcohol, and stained by immunohistochemistry with specific anti-JEV NS3 monoclonal antibody. The results showed that the NS3 protein was expressed in brain neuronal cells and fat body, both in *Aedes* and *Culex* mosquitoes. For comparison, JEV-positive infection of adult mosquitoes after intrathoracic injection with 300 pfu viruses for 5 days is also shown in Figure 5b (left panels).

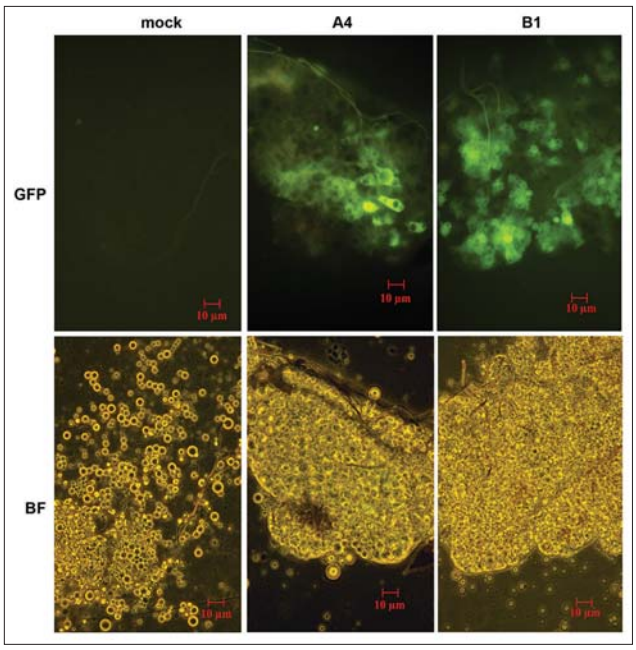

Figure 4: GFP was expressed in fat bodies of adult female mosquito *in vivo*. Female adult *Aedes aegypti* were microinjected with pGL3-A4-GFP and pGL3-B1-GFP plasmids (98 nl containing ~ 50 ng DNA per mosquito) left for 5 days. Then, the fat bodies were microdissected on slides and examined by using a Zeiss Axio Scope A1 fluorescent microscope; images were captured by a Canon EOS 450D digital system. The upper panels show fluorescence detection; the lower panels are bright field images. Scale bar = 10 mm

DISCUSSION

Ae. aegypti transmits viruses causing dengue fever, yellow fever, chikungunya fever, and Zika fever, which are among the greatest public health burdens in many countries. The main reasons for this dire situation are the unavailability of effective antivirus vaccines (except for yellow fever), lack of therapeutic drugs, development of insecticide resistance in mosquitoes, and unsuccessful vector control. Therefore, there is an urgent need to explore other possible avenues for developing novel control strategies against these mosquito-borne viruses, such as by genetically engineering mosquitoes to have pathogen-resistant phenotypes, or by using molecular approaches in mosquito itself to identify and neutralize potential viral gene targets that counteract mosquito immune competence. Generation of pathogen-resistant or immunocompetent transgenic mosquitoes requires a promoter that can drive the expression of effector molecules, ectopic genes, siRNA, or viral genes in an appropriate manner.[2,4,20-22]

We showed here that the promoters from the AMP genes – defensin A1, defensin A4, and cecropin B1, but not the Hsp promoter, could effectively drive constitutive expression of luciferase, GFP, and glycosylated G protein of rabies virus in cell lines without external stimulation such as heat shock. These

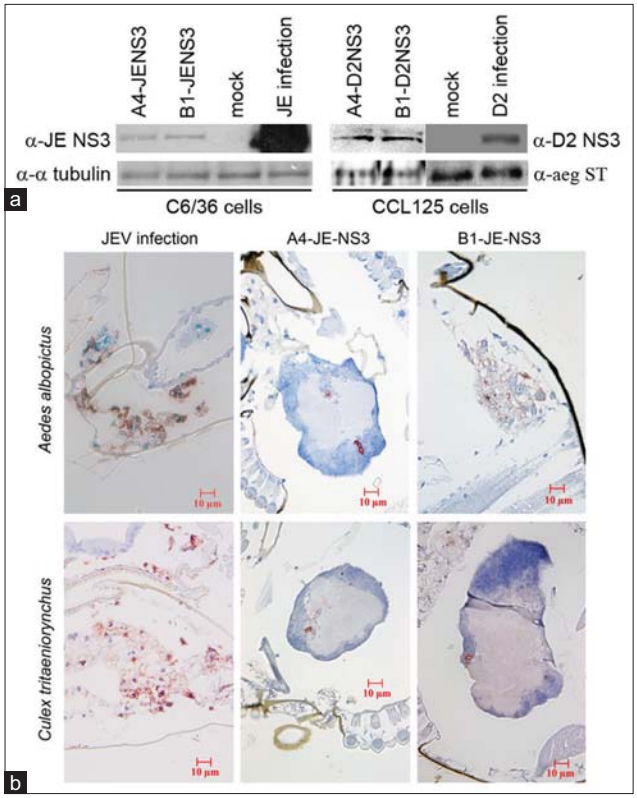

Figure 5: (a) The viral protein NS3 of JEV and DenV2 can be expressed in mosquito cells under the control of A4 and B1 promoters. Plasmids – pGL3-A4-JENS3, pGL3-B1-JENS3, pGL3-A4-D2NS3, and pGL3-B1-D2NS3 were transiently transfected into C6/36 and CCL125 mosquito cells for 48 h. The cells were lysed and the lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane and probed with monoclonal antibodies against NS3 of JEV and DenV2. C6/36 and CCL125 cells were also infected with JEV or DenV2 virions as a positive control. (b) The JEV NS3 gene alone can be expressed in adult mosquitoes. *Aedes albopictus* (upper panels) and *Culex tritaeniorhynchus* (lower panels) adult were intrathoracically injected with pGL3-A4-JENS3 or pGL3-B1-JENS3 (98 nl containing ~50 ng DNA per mosquito) and left for 5 days. The injected mosquitoes were formalin fixed and paraffin embedded, and 5-microm sliced sections were dewaxed in xylene, rehydrated through graded alcohol, and stained by immunohistochemistry with specific anti-JEV NS3 antibody. The brown/red areas indicate positive NS3 protein expression in mosquito brain neuronal cells and fat body. The left panels show JEV-infected tissue as a positive control. Scale bar = 10 μm

novel promoters could also drive both flavivirus NS3 and GFP expression in adult mosquito tissues, including neuronal cells and fat body of *Ae. aegypti*, *Ae. albopictus*, and *Cx. tritaeniorhynchus in vivo* without external induction such as blood feeding.

The innate immune response of *Ae. aegypti*, like that of *Drosophila* and other insects, can protect the mosquito from invasion by pathogens through rapid and potent production of immune peptides, including defensins, cecropins, and several other AMPs which are secreted into the hemolymph.[23-29] The promoters of these insect AMP genes contain several functional domains, such as kappaB-like motifs, GATA motifs, C/EBP binding sites, or acute-phase response elements, which are very similar to those of mammalian promoters.[30-35] The immune response mediated by these defense genes is induced by the Toll and/or Imd signaling pathways through binding the downstream transcription factors – Dorsal and Relish, respectively, to regulatory elements of AMP genes, thereby activating transcription and secretion of immune peptides against fungi, Gram-positive, and Gram-negative bacteria and restrict virus replication.[36-43]

Tzou *et al*. used several promoters from *Drosophila* AMP genes to construct GFP reporter transgenes and demonstrated that AMPs can be induced in surface epithelia in a tissue-specific manner, and some promoters were constitutively active in various regions of particular tissues.[44] The NF-kappaB transcription factor plays a major role in the induction of AMP expression, and the *Drosophila* homeobox gene caudal functions as immune transcription modulator for constitutive expression of AMPs.[45] AMPs were demonstrated to be expressed constitutively in the malpighian tubules of *Drosophila*, which function as autonomous immune sensing organs.[46] Accordingly, in our experiments which demonstrated constitutive expression of GFP, luciferase, NS3, or G protein under the control of the A4 and B1 promoters without external induction, the genes may be regulated by transcription factors or homeobox-containing proteins other than, or in addition to, NF-kappaB. Expression of GFP by the A4 or B1 promoter in the fat body of adult female mosquitoes *in vivo* [Figure 4] suggest that this main AMP producing organ, equivalent to the mammalian liver, could respond to microbe invasion by secreting AMPs into the hemolymph.[25]

The G protein of rabies virus induces neutralizing antibodies, which are important in protection against rabies. Following the work of Dos Santos *et al*., who used the *Drosophila* actin promoter to drive rabies G protein expression in S2 cells,[47] we constructed a B1 promoter to successfully drive rabies G protein production in a mosquito cell line [Figure 2d].

Cao *et al*. recovered a mutant of dnr1, a negative regulator of the Imd immune pathway, and found this mutant to exhibit neurodegeneration and elevated expression of AMP genes.[48] Petersen *et al*. demonstrated an association between the innate immune response and neurodegeneration in a *Drosophila* model of the human disease Ataxia–telangiectasia, with elevated expression of innate immune response genes in glial cells.[49,50] We showed ectopic expression of the NS3 protein of JEV not only in the fat body but also in brain neuronal cells of two species of mosquito [Figure 5b], and suspected that AMP genes could be implicated in mosquito brain inflammation.

Our finding that the promoters of mosquito AMP genes are able to drive expression of luciferase or GFP proteins in insect cells [Figures 1 and 3], NS3 proteins of JEV or DenV2 in both mosquito cell lines and adult mosquitoes [Figure 5], G protein of rabies virus in mosquito cells [Figure 2d], and NS3 protein

in fat body [Figure 4] and neuronal cells of *Ae. albopicus* and *Cx. tritaeniorhynchus* [Figure 5b], indicates that these mosquito promoters (especially A4 and B1) are robustly active and evolutionarily conserved within the mosquito lineage and could be used for exogenous protein expression in mosquito species *in vitro* and *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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