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A viral over-expression system for the major malaria mosquito *Anopheles gambiae*

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Understanding pathogen/mosquito interactions is essential for developing novel strategies to control mosquito-borne diseases. Technical advances in reverse-genetics, such as RNA interference (RNAi), have facilitated elucidation of components of the mosquito immune system that are antagonistic to pathogen development, and host proteins essential for parasite development. Forward genetic approaches, however, are limited to generation of transgenic insects, and while powerful, mosquito transgenesis is a resource- and time-intensive technique that is not broadly available to most laboratories. The ability to easily "over-express" genes would enhance molecular studies in vector biology and expedite elucidation of pathogen-refractory genes without the need to make transgenic insects. We developed and characterized an efficient *Anopheles gambiae* densovirus (AgDNV) over-expression system for the major malaria vector *Anopheles gambiae*. High-levels of gene expression were detected at 3 days post-infection and increased over time, suggesting this is an effective system for gene induction. Strong expression was observed in the fat body and ovaries. We validated multiple short promoters for gene induction studies. Finally, we developed a polycistronic system to simultaneously express multiple genes of interest. This AgDNV-based toolset allows for consistent transduction of genes of interest and will be a powerful molecular tool for research in *Anopheles gambiae* mosquitoes.

Anopheles sp. are the only mosquitoes that transmit *Plasmodium* parasites to humans, and as such, are a major concern for public health[1]. *Anopheles gambiae* is the major vector of *Plasmodium falciparum*, the major cause of malaria in sub-Saharan Africa[2]. Issues with current control strategies such as the lack of effective vaccines and emergence of insecticide resistance[1,3] necessitate the need for novel disease prevention measures. One such strategy is to control pathogen transmission by mosquitoes using transgenic technology. However, generation of transgenic *Anopheles* mosquitoes is technically challenging, and limited success has been reported in *An. gambiae*[4–6]. Alternative strategies such as the use of microbes to control pathogens are gaining considerable attention (reviewed in refs)[7–9].

Paratransgenesis (genetic manipulation of symbiotic microorganisms to interfere with pathogen development in the host) has been proposed as a control strategy for vector-borne diseases[10]. Paratransgenic control approaches have several advantages over strategies based on insect transgenesis. Microorganisms help maintain host homeostasis can be tightly associated with their insect host. These microbes are often more straightforward to transform compared to the insect[11], and microbes can often spread through insect populations[12–15]. As such, paratransgenic control strategies are being considered for a wide range of viral, bacterial and fungal microbes[16–25]. Paratransgenic approaches using bacteria and fungi have been shown to significantly reduce *Plasmodium* levels in *An. gambiae*[18,20].

Pathogens within mosquitoes can also be inhibited by manipulating host genes essential for pathogen development[26–28]. To explore such gene functions, effective tools to manipulate the host are essential. However manipulating gene expression in *Anopheles* mosquitoes has been constrained because only a few techniques are available, mainly RNA interference (RNAi) and transgenic manipulation[10,29–31]. As such, the development of efficient and simple transient over-expression systems in mosquitoes will facilitate investigations on mosquito biology and applied mosquito-borne diseases control strategies.

Viral vector transduction is a common approach to over-express genes in many host systems (reviewed in refs)[32–35]. Viruses actively enter target cells, are effective *in vitro* and *in vivo* and can be modified for specific aims such as tissue tropism[36,37]. Densoviruses (DNVs) are non-enveloped single-stranded parvoviruses that are widely

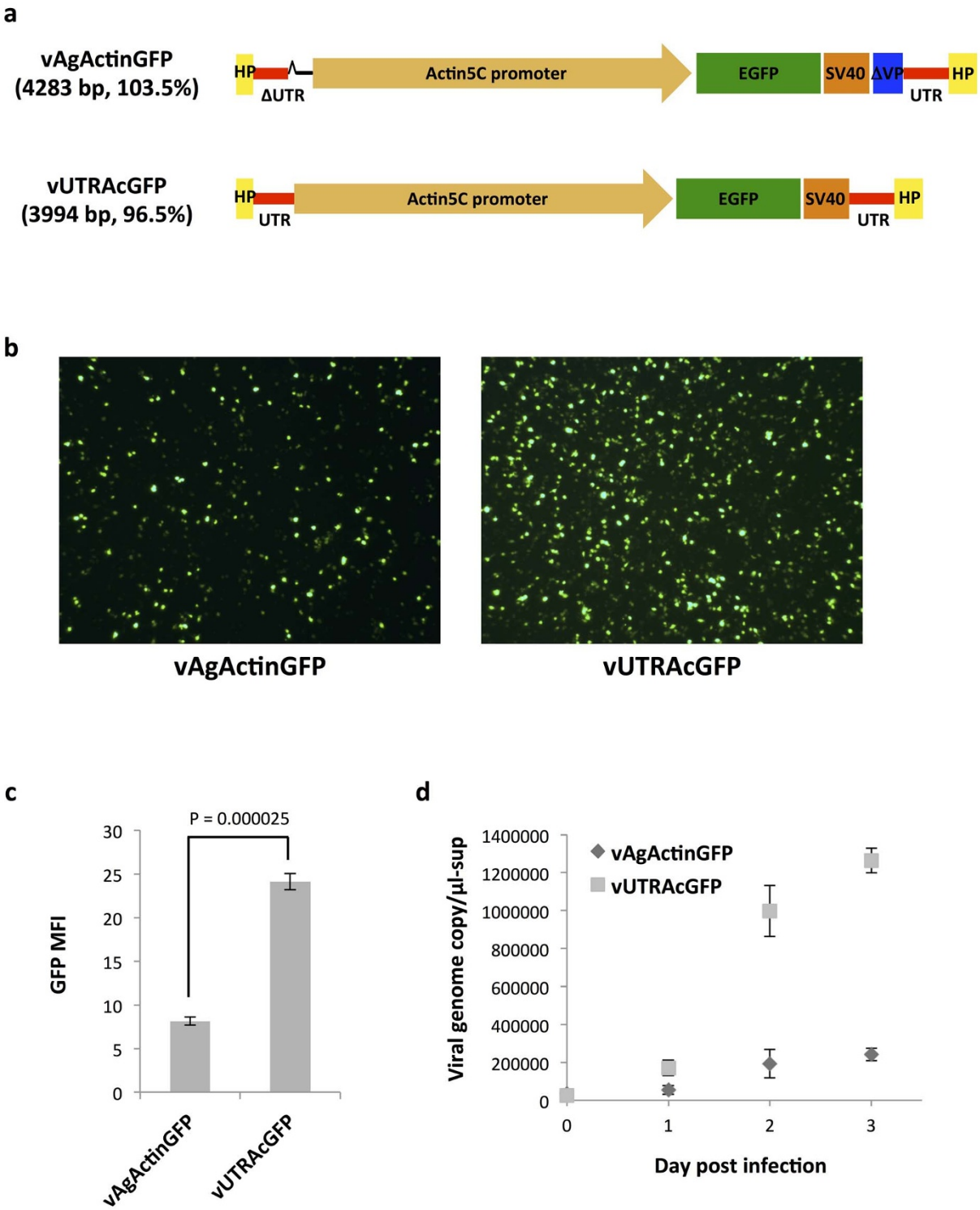


Figure 1 | New AgDNV transducing vector. (a) Schematic representation of recombinant AgDNV vectors. Both vectors consist of the viral terminal hairpins, UTR's and EGFP gene driven by Actin5C promoter and SV40 terminator. vAgActinGFP lacks 27 bp of the 5′ UTR (referred to as ΔUTR) and contains a portion of the viral capsid gene (ΔVP). vUTRAcGFP has the intact 5′ UTR and no viral capsid gene sequence. Each vector genome size relative to wild-type virus is indicated. (b) MOS55 cells were infected with equivalent titers of vAgActinGFP or vUTRAcGFP. EGFP expression was visualized by fluorescence microscopy. (c) The mean fluorescence intensity (MFI) of EGFP was determined by flow cytometry. (d) Recombinant AgDNV replication in MOS55 cells. Supernatants from infected cells were collected from Days 0–3 post infection. Viral DNA was quantified by qPCR. Mean and standard deviation (S.D.) were calculated from three independent infections in (c) and (d). Treatments are significantly different (Student's T test).

distributed among arthropods including multiple mosquito species[38–43]. *Aedes aegypti* densovirus (AeDNV) has been intensively studied as a transducing vector for *Aedes* mosquitoes[44–47]. DNVs are often pathogenic to mosquitoes[45,48], and their pathogenicity can be improved by engineering[46]. Previously, we isolated a DNV from *Anopheles gambiae* (AgDNV), showed that it replicates preferentially in adult mosquitoes, imparts minimal impact on host genes expression and is avirulent[19,49,50]. Here we report on the development of an

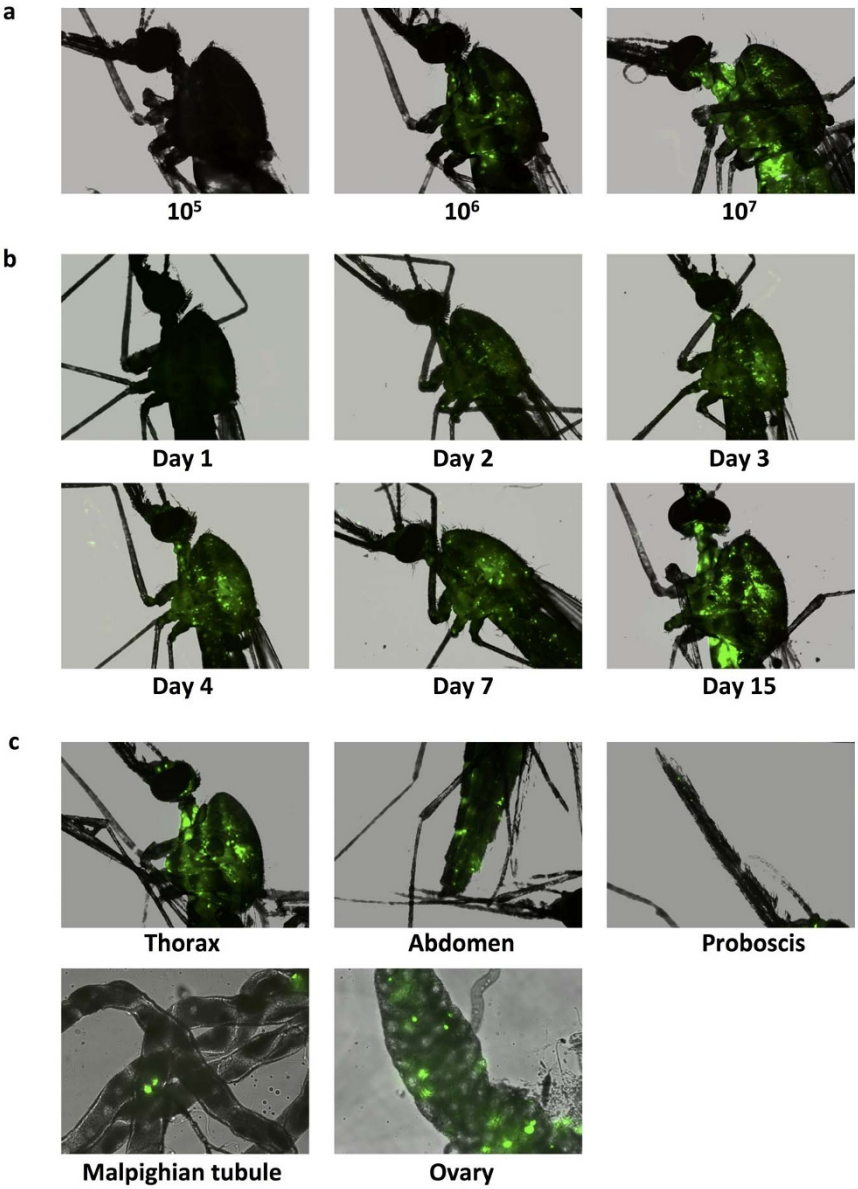


Figure 2 | Transduction of *An. gambiae* with vUTRAcGFP by intrathoracic microinjection. EGFP expression was visualized by fluorescence microscopy. (a) *An. gambiae* adults were injected with 1×10^5, 10^6 or 10^7 particles of vUTRAcGFP and visualized at seven days post-injection. (b) Non-destructive time course of EGFP expression in the mosquitoes injected with 1×10^7 of vUTRAcGFP. (c) Tissue tropism of recombinant AgDNV. Fluorescence was observed in the fat body, ovaries, malpigian tubules and proboscis.

improved viral transduction system, which can efficiently overexpress multiple genes of interest in *An. gambiae* mosquitoes at high frequency.

Results

Generation and evaluation of a new recombinant AgDNV vector. We use the prefix "v" to denote viral vectors and "p" to indicate their infectious plasmids. Our previous recombinant AgDNV harboring the EGFP gene, vAgActinGFP (derived from pAgActinGFP) is 4283 base pairs (bp) in length and is approximately 3.5% longer than the wild-type AgDNV genome[19]. In the course of working with this virus for several years, we noted that EGFP expression in *An. gambiae* adults infected with vAgActinGFP is highly variable (unpublished observation). The variation is possibly due to the large size of the viral genome. For other DNVs, efficient packaging of the viral genome is inhibited when the transducing genome was larger than the wild-type genome[51]. To shorten our recombinant AgDNV vector, we generated a new DNV vector plasmid (pUTR) which contained both hairpins and the entire AgDNV 5′ and 3′ untranslated regions without any ORFs from the wild-type AgDNV plasmid pBAgα[19]. The Actin5C promoter-EGFP-SV40 terminator cassette was inserted into pUTR and a new transducing construct developed (pUTRAcGFP). vUTRAcGFP has a genome length of 4011 bp, which is 128 bp shorter than wild-type AgDNV genome (4139 bp). We compared transduction and replication efficiency between vUTRAcGFP and vAgActinGFP (Fig. 1a). To provide viral proteins for replication of recombinant viruses, all recombinant virus samples were prepared by co-transfection of pBAgα (wild-type AgDNV plasmid) and recombinant virus plasmids. MOS55 cells were infected with equal titers of the vAgAcGFP or vUTRAcGFP. vUTRAcGFP-infected cells showed 3-fold higher intensity of EGFP than cells infected with vAgAcGFP (Fig. 1b and c). To compare the replication kinetics of each viral vector, supernatant was collected from the DNV-infected MOS55 cells at 0–3 days post infection and the encapsulated recombinant

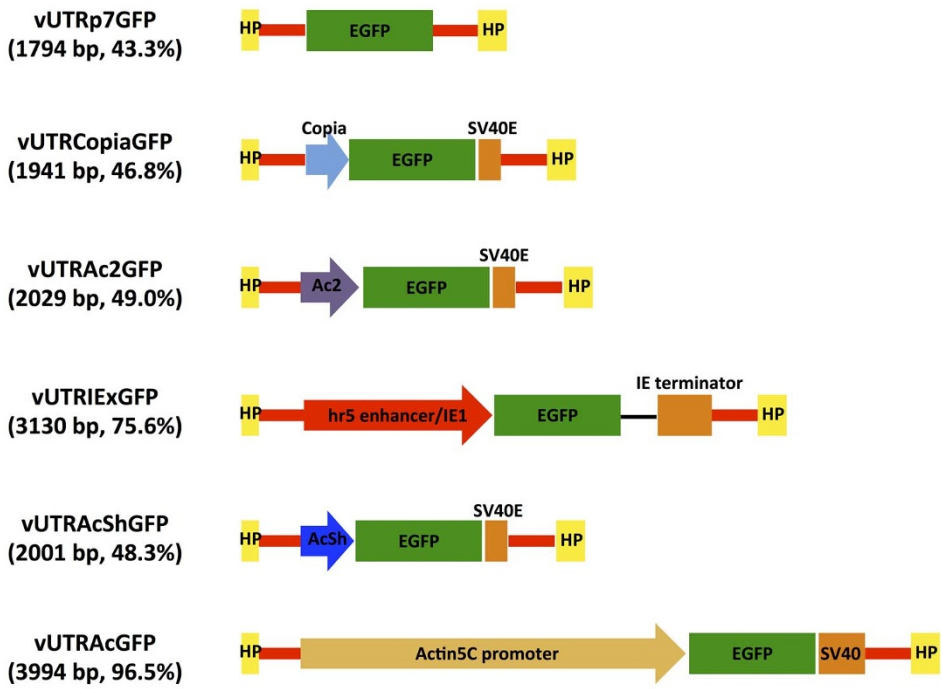


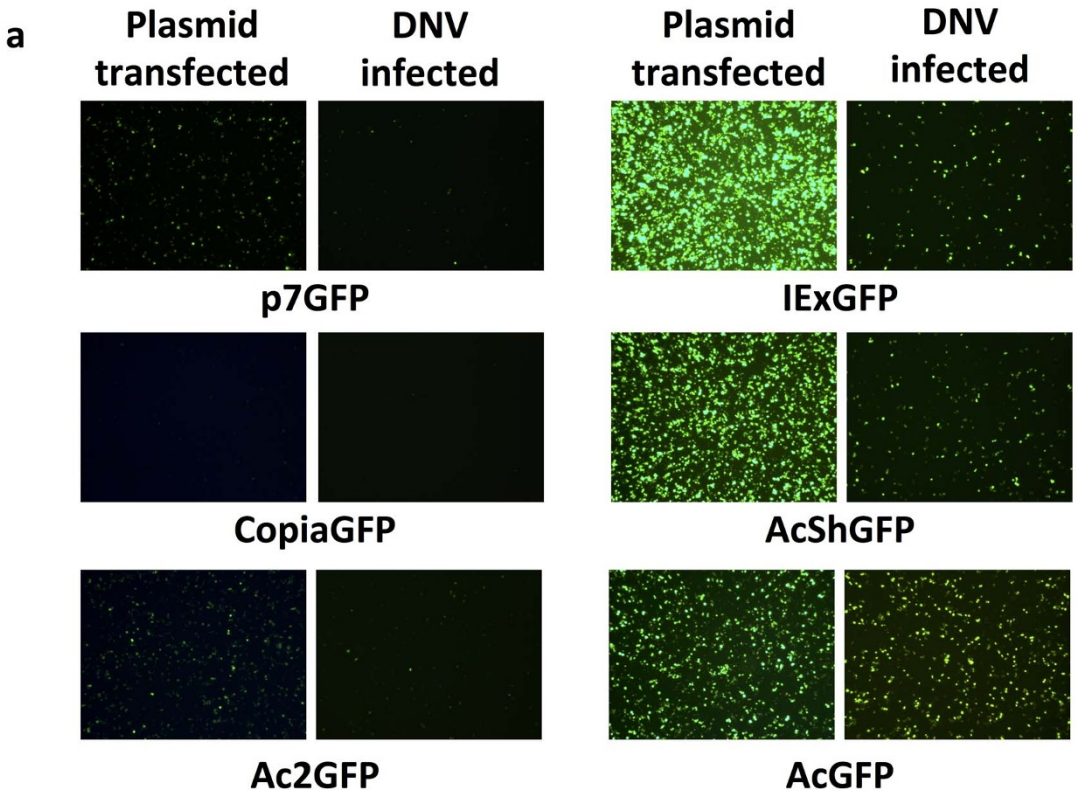
Figure 3 | Schematic representation of constructed recombinant AgDNV vectors harboring different promoter sequences. Each vector genome size ratio relative to wild-type virus is indicated.

viral genome DNA enumerated by quantitative PCR (qPCR). The recombinant viral DNA of vAgActinGFP plateaued at approximately 2–2.5 $\times$ 10^5 genome copies per µl after two days. In comparison, vUTRAcGFP showed over a 5-fold increase of 1.2 $\times$ 10^6 copies per µl at the same time point and had not yet plateaued (Fig. 1d).

High level and consistent transduction of AgDNV in *An. gambiae* mosquitoes. Next, we assessed the infectivity of vUTRAcGFP *in vivo*. Recombinant virus and helper virus was intrathoracically injected into the thorax of adult female mosquitoes, with 10–20 mosquitoes per treatment. Adult mosquitoes were injected with 1 $\times$ 10^5, 10^6 or 10^7 vUTRAcGFP and collected 7 days post injection to examine EGFP expression. Fluorescence was observed in all mosquitoes in a dose-dependent manner (Fig. 2a). We next conducted a virus replication time course experiment, repeatedly non-destructively examining the same mosquito at each time point. EGFP was visible beginning day two post-injection. Fluorescence increased throughout the experiment to 15 days post-injection (Fig. 2b). To determine the tissue tropism of vUTRAcGFP, we dissected infected mosquitoes. The majority of EGFP expression was observed in the fat body and the ovary (Fig. 2c), but was also visible in other tissues including muscle, malpighian tubules and the proboscis (Fig. 2c). No significant fluorescence was observed in midgut or salivary glands (data not shown).

Comparison of 6 different promoter cassettes for recombinant AgDNV gene transduction. Parvoviral vectors have a size limitation for efficient genome packaging. In *Aedes aegypti* DNV-based vectors, genomes that exceed the wild-type size by more than 10% cannot be efficiently packaged[51]. pUTR with the Actin5C promoter cassette is 3291 bp in length, allowing for the insertion of genes less than 1000 bp. It would be desirable to shorten the promoter so that larger genes of interest can be expressed in this system. We assessed several shorter promoters by exchanging the Actin5C promoter in pUTRAcGFP. All viruses were compared for their ability to transduce *Anopheles gambiae* both *in vitro* and *in vivo*. All recombinant DNV genomes contained the EGFP gene under the control of different promoters: vUTRp7, the native AgDNV non-structural promoter and polyadenylation sequence that exist in the UTR sequences; vUTRCopia, 247 bp copia promoter and 134 bp SV40 early polyadenylation signal (SV40E polyA); vUTRAc2, 335 bp Actin5C promoter for exon2 and exon3[52] and SV40E polyA; vUTRIEx, 1082 bp *Autographa californica* nuclear polyhedrosis virus (AcNPV)-derived hr5 enhancer and immediate early promoter IE1 and 308 bp IE terminator; vUTRAcSh a 307 bp truncated version of the Actin5C promoter[53] and SV40E polyA (Fig. 3). We evaluated activity of each promoter cassette in plasmid-transfected and virus-infected MOS55 cells (Fig. 4a). In plasmid-transfected cells, the IEx promoter showed the highest level of EGFP expression (Fig 4a). In pUTRAcShGFP-transfected MOS55 cells, relatively high expression was observed (Fig 4a). Both of IEx and AcSh promoter cassettes showed higher expression of EGFP than the longest Actin5C promoter. In comparison, transduction experiments with virus demonstrated that vUTRAcGFP had higher transduction efficiency than vUTRIExGFP or vUTRAcShGFP (Fig. 4a). We quantified viral promoter efficiency using flow cytometry. Viruses driving EGFP from p7, copia or Ac2 showed very little fluorescence. In contrast, viruses containing the IEx, short actin or long actin promoters showed strong fluorescence. The Actin5C promoter had 21 times higher EGFP intensity than the p7 promoter ($P < 0.00005$) (Fig. 4b). IEx and AcSh promoters showed 4.4 and 4.1 times higher activity than p7 respectively ($P < 0.005$) (Fig. 4b). Next, we compared EGFP transduction *in vivo*. *An. gambiae* adults were injected with 5 $\times$ 10^6 of each of the recombinant DNVs and EGFP expression was visualized at day 7 post-injection. Similar to results *in vitro*, vUTRAcGFP-transduced mosquitoes showed the highest EGFP expression (Fig. 5). Although the intensities were lower than Actin5C, intermediate EGFP expression was observed in vUTRIExGFP- and vUTRAcShGFP-infected mosquitoes. No visible fluorescence was observed in the mosquitoes infected with vUTRP7GFP, vUTRCopiaGFP or vUTRAc2GFP.

Development of a polycistronic AgDNV-based expression vector. vUTRAcGFP harbors the longest promoter-cassette and showed the



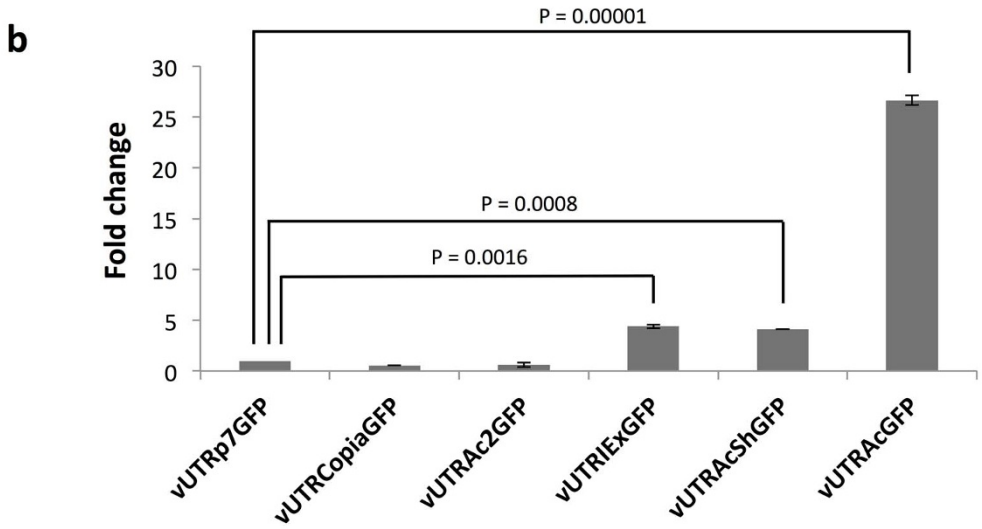


Figure 4 | Comparison of expression promoter constructs *in vitro*. (a) EGFP expression visualized by microscopy in plasmid-transfected and DNV-infected MOS55 cells. 2.5 μg of plasmids and 5×10^8 of recombinant DNVs were used for transfection and infection experiments respectively. (b) EGFP intensity was quantified by a flow cytometry in each of the DNV-infected MOS55 cell cultures. Fold-changes in EGFP expression was compared to vUTRp7GFP-infected cells. Mean and S.D. were calculated from three independent infections. P-values were calculated by Student's T test.

highest expression of EGFP among the investigated AgDNV vectors. However, the smaller vUTRIExGFP and vUTRAcShGFP had intermediate expression activity *in vitro* and *in vivo* (Fig. 4 and 5). We chose the recombinant AgDNV containing the AcSh promoter as a backbone as it could accommodate up to 2.7 kb of cargo. To polycistronically express multiple open reading frames we used the *Thosea asigna* virus-derived 2A-like sequence[54,55] to simultaneously express 3 genes; mCherry, EGFP and the AgDNV viral capsid protein (Fig. 6, vUTRAcShmCherry-GFP-VP). Western blot for EGFP confirmed that the 2A-like sequences were cleaved in mosquito cells (Fig. 7a, Supplementary Fig. 1). mCherry and EGFP fluorescence was visually observed in vUTRAcShmCherry-GFP-VP-infected MOS55 cells (Fig. 7b) and quantified using flow cytometry (Fig. 7c). The percentage and intensity of EGFP-positive cells were significantly higher than those infected with vUTRAcShGFP-infected cells ($P < 0.005$ and $P < 0.0005$ respectively) (Fig. 7d). *An. gambiae* adults were injected with 5×10^6 of each recombinant DNV. Both mCherry and EGFP fluorescence was detected in

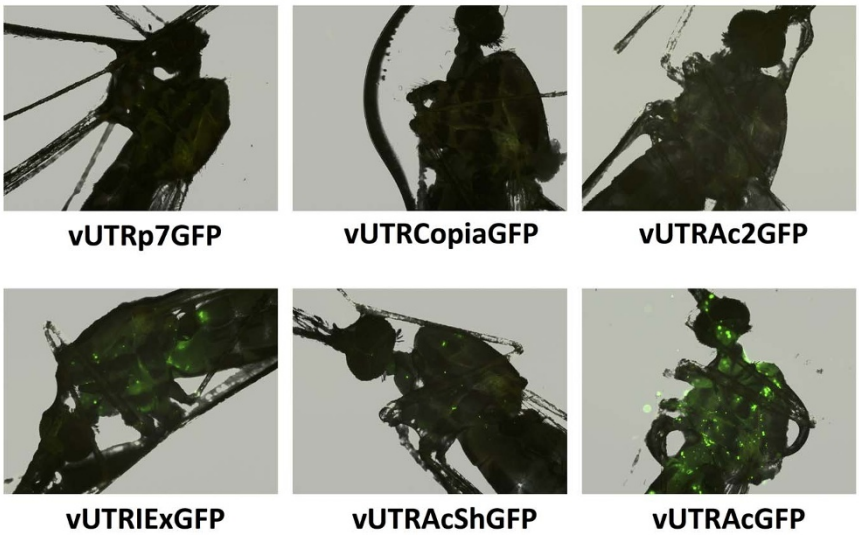


Figure 5 | Comparison of different promoter efficiency for AgDNV transduction *in vivo*. *An. gambiae* adults were injected with 5×10^6 of each recombinant DNV. EGFP expression in mosquitoes was examined at seven days post-injection.

vUTRAcShmCherry-GFP-VP-infected mosquitoes (Fig. 8). Again, EGFP expression was higher in vUTRAcShmCherry-GFP-VP-infected compared to vUTRAcShGFP mosquitoes (Fig. 8) even though mosquitoes in both treatments were infected with the same amount of virus. Levels of expression could also be boosted in a dose-dependent manner (Fig. 8).

Discussion

Molecular tools to manipulate gene expression have drastically changed the scientific landscape of vector biology[56–58]. Engineering mosquitoes with enhanced immunity is a potential approach for the control of mosquito-borne diseases[59,60]. However, over-expression of host genes has not been widely employed due to the lack of efficient and convenient approaches[61]. Our previous recombinant AgDNV vector could transduce *An. gambiae* adults, but transduction efficiency was highly variable. A new smaller vector containing the complete viral UTR sequence, (vUTRAcGFP) had 3-fold higher transduction efficiency *in vitro* compared to our previous vector (vAgActinGFP). In infected cells, vUTRAcGFP showed more efficient replication than vAgActinGFP. Within parvoviruses, the viral genome size significantly affects packaging and replication[51,62]. Afanasiev *et al.* suggested that longer DNV genomes were packaged less efficiently than shorter ones[51]. Other studies demonstrated that a specific region of viral DNA including the 5′ UTR was important for efficient AAV production[63]. Taken together, these studies suggest shortening the recombinant viral genome and complementing the complete 5′ UTR sequence may synergistically increase transduction efficiency of vUTRAcGFP. In the mosquito, high-levels of GFP expression lasted over fifteen days indicating that AgDNV-based transduction system can efficiently over-express genes for the entire lifespan of the mosquito. By visual observation, there was minimal variation in the intensity detected among individuals.

The most prolific vector in terms of EGFP expression had a relatively long promoter (Actin5C) allowing the insertion of genes approximately 800 bp. We compared the activities of several shorter promoters. In plasmid-transfected cells, the IEx and AcSh promoters showed higher expression than Actin5C (Fig. 4a). Conversely, vUTRAcGFP transduced both the cell line and the mosquito more efficiently than other transducing viruses. The fluorescence intensity in transduced cells and mosquitoes should reflect not only promoter activity but also viral replication. Tullus *et al.* demonstrated that efficient production of single-stranded AAV DNA that can be packaged into the virion needs a certain minimal size[63]. AgDNV may also have a limitation on the minimum genome DNA size required for the efficient replication. The polycistronic DNV vector under the control of AcSh promoter, which has a similar size to the wild-type AgDNV, showed higher level of EGFP expression than the much shorter vUTRAcShGFP in cell cultures and mosquitoes. Taken together, recombinant AgDNV genome size may be involved in replication efficiency. Future analyses will address the detailed requirements and mechanisms for generation of efficient AgDNV vectors.

By EGFP expression, AgDNV infection was primarily visible in the fat body. The mosquito fat body plays crucial roles in mosquito physiology, including energy storage, metabolism and immunity. This tissue is also proximal to *Plasmodium* parasites which migrate throughout the hemocoel to invade the salivary glands[64,65].

The other main target tissue of AgDNV was the ovary. Infection of the ovary may be involved in potential vertical transmission of the virus or inoculation of virus into the larval habitat during oviposition. Although significant transduction was not observed in midgut

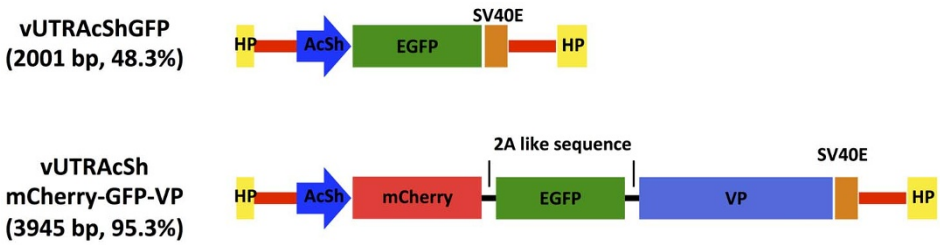


Figure 6 | Schematic representation of polycistronic AgDNV vector. vUTRAcShmCherry-GFP-VP and its backbone vector vUTRAcShGFP genomes are illustrated. Each vector genome size ratio relative to wild-type virus is indicated.

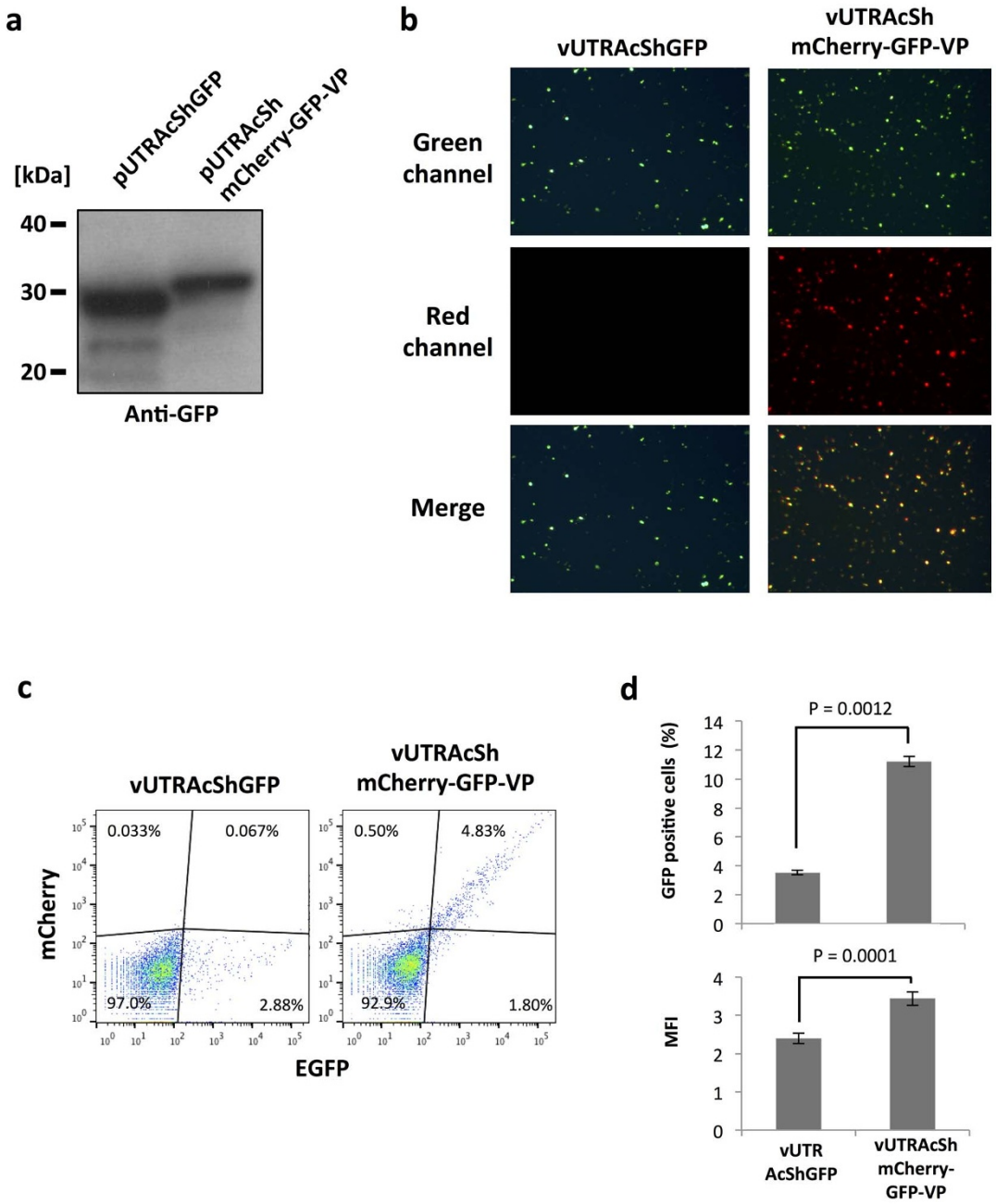


Figure 7 | Multiple gene transduction by a polycistronic AgDNV vector *in vitro*. (a) Western blot analysis of pUTRAcShGFP- and pUTRAcShmCherry-GFP-VP-transfected MOS55 cells with anti-GFP antibody. Samples were run on the same gel under identical conditions. Masses of protein size standards were indicated at left. Blot has been cropped; full uncropped blot with duplicates available as Supplementary Figure 1. MOS55 cells were infected with 5×10^8 of each vUTRAcShGFP and vUTRAcShmCherry-GFP-VP. EGFP and mCherry expression was (b) visualized by fluorescence microscopy and (c) quantified by flow cytometry analysis. (d) The rate of EGFP positive cells (upper panel) and MFI (lower panel) was quantified by flow cytometry. Mean and S.D. were calculated from three independent infections. P-values were determined by Student's T test.

and salivary gland (which are important tissues for *Plasmodium* infection) transduction of the fat body and ovary suggest promising strategies to use AgDNV to control *Anopheles*-transmitted parasites. For example, anti-*Plasmodium* peptides such as SM1 or scorpine would be desirable molecules to express as they are short enough to insert into the current AgDNV vector. High expression in the fat body and secretion into the hemolymph has the potential to dramatically inhibit *Plasmodium*[18,20,66,67]. Using the polycistronic AgDNV vector multiple anti-*Plasmodium* genes could be expressed simultaneously, leading to a synergistic effect on the parasite. In addition, transduction in the ovaries opens the possibility of using AgDNV to manipulate *Anopheles* reproduction.

In conclusion, we have developed an efficient AgDNV-based overexpression system for *Anopheles gambiae* mosquitoes. Our data suggested that viral genome size and the 5′ UTR sequence are important elements for efficient AgDNV infection and replication. These insights may shed light upon other insect parvovirus life cycles, which are less well known. Further studies will expand applications to investigation of mosquito biology and paratransgenesis for malaria control.

Methods

Cells culture and mosquito rearing. *An. gambiae* MOS55 cells were maintained in Schneider's medium supplemented with 10% fetal bovine serum at 28°C. *An. gambiae*

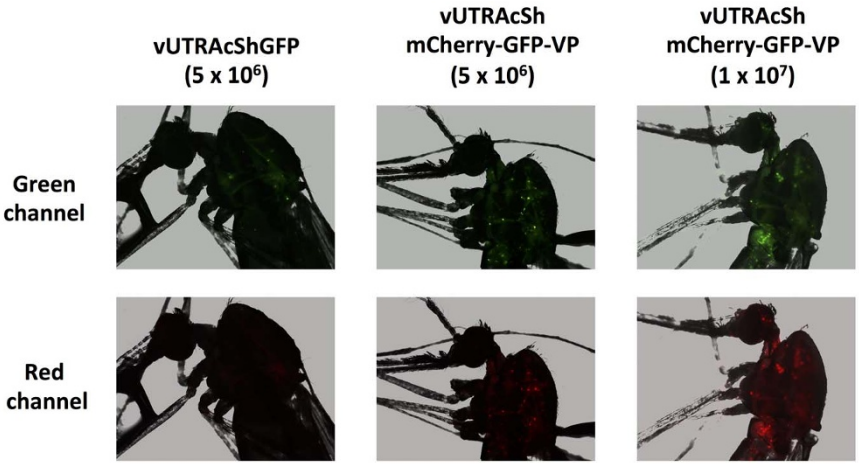


Figure 8 | Multiple gene transduction by a polycistronic AgDNV vector *in vivo*. *An. gambiae* adults were injected with 5×10^6 or 1×10^7 of each recombinant DNV. EGFP and mCherry expression in the mosquitoes was visualized at seven days post-injection.

mosquitoes (Keele strain) were maintained on 10% sugar solution at 28°C and 80% humidity with 12/12 h light/dark cycle. The larvae were fed with tetramin fish food. Adults were offered expired human blood through a membrane feeder for reproduction.

Plasmid construction. The pUTR vector was generated based on pBAgα[19] (infectious clone of AgDNV) by PCR using the forward primer (5′-ATA-TTT-TAA-TCA-ACA-TGT-ATC-AAC-TAT-A-3′) and reverse primer containing an EcoRV site (5′ GAT-ATC-CAC-TCA-ATT-CGC-CTC-TCC-TTT-TG 3′). The Actin5C promoter-EGFP-SV40 terminator cassette from pAgActinGFP[19] was inserted into the EcoRV site in pUTR to make pUTRAcGFP. pUTRp7GFP was constructed by inserting the EGFP ORF into pUTR. The copia-SV40E, AcSh-SV40E and Ac2-SV40E cassettes were commercially synthesized (Integrated DNA Technologies) and inserted into the pUTR vector with the EGFP gene. The resulting vectors were referred to pUTRCopiaGFP, pUTRAcShGFP and pUTRAc2GFP respectively. To clone the AcNPVhr5 enhancer-IE1 promoter and IE1 terminator, pIEx4 (Novagen) was digested with SmaI/ZraI. pUTRIExGFP was made by ligating pUTR and the resulting fragment with the EGFP gene. The polycistronic vector pUTRAcShmCherry-GFP-VP was constructed based on pUTRAcSh, which contains the short actin promoter and multiple cloning site. The AgDNV VP gene was amplified from pBAgα and cloned into the BglII/MluI site in pUTRAcSh. The mCherry-T2A-GFP-dT2A sequence was obtained by PCR from pAc5-STABLE1-Neo (Addgene)[54] and inserted into the BglII site in pUTRAcShVP. SURE2 Competent cells (Stratagene) were used for all cloning strategies, and all plasmids confirmed by direct sequencing.

Densovirus production. MOS55 cells at 70% at confluence in a 6-well plate were transfected with each recombinant AgDNV plasmid and the helper plasmid pBAgα at a ratio of 2 : 1 (1.67 μg and 0.83 μg respectively) using Lipofectamine LTX reagent (Life Technologies). At 3 days post transfection, cells were harvested and suspended in PBS. The cell suspension was subjected to freezing-thawing three times and cell debris was removed by centrifugation. The supernatant was used as densovirus samples for DNA extractions.

Quantification of Densovirus DNA by real-time PCR. Recombinant DNV samples were treated with TURBO DNase (Ambion) to digest plasmid DNAs. The DNase-resistant viral DNA number was considered as the number of recombinant virions. Total DNA from the densovirus samples or densovirus-infected cell supernatants was extracted using DNEasy kits (Qiagen) according to the manufacturer's suggested protocol. Quantitative PCR was performed using Quantitect SYBR Green Kit (Qiagen) using a Rotor-Gene Q (Qiagen). The following primer pairs were used for amplification of EGFP gene: EGFP forward: 5′ TCA-AGA-TCC-GCC-ACA-ACA-TC 3′, EGFP reverse: 5′ TTC-TCG-TTG-GGG-TCT-TTG-CT 3′. To quantify recombinant DNV genome copy number, standard curve was created from a dilution series of pUTRAcGFP ranging from 10^3 to 10^8 copies.

Transduction of *Anopheles gambiae* cell lines and mosquitoes. MOS55 cells were infected with recombinant DNV sample corresponding to 5×10^8 of recombinant virus. For quantification of the viral DNA genome, the supernatant of infected cells was collected at indicated days after infection. EGFP expression in the cell line was observed using an Axiovert S100 fluorescence microscope (Zeiss) and images captured using a ProgRes CF camera (Jenoptik). EGFP signal was quantified by flow cytometry on a Cytomics FC500 (Beckman Coulter). Data were analyzed using BD LSRFortessa (BD Biosciences) and FlowJo software. *Anopheles gambiae* adults were intrathoracically injected according to Hughes et al. (2011)[68] with the indicated viral copy number of recombinant DNV virions. Fluorescence in mosquitoes and dissected tissues was monitored at indicated days post-injection using an Olympus BX-41 microscope. Images were processed using PictureFrame software (Olympus).

Western Blotting. Plasmid-transfected MOS55 cells were lysed in Laemmli Sample Buffer (BioRad) containing 2.5% of β-mercaptoethanol. Protein samples were separated by SDS-PAGE on 4–15% Mini-PROTEAN® TGX™ Gel (BioRad). EGFP was detected with anti-GFP polyclonal antibody (Santa Cruz Biotechnology) and anti-rabbit IgG HRP-liked antibody (Cell Signaling). Signals were developed on Amersham Hyperfilm ECL (GE healthcare) with Amersham ECL Plus Western Blotting Detection Reagents (GE healthcare).

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Author contributions

Y.S. designed and performed the experiments, analyzed the data and contributed to drafting the manuscript. G.N. provided technical support for experiments. G.L.H. assisted in data analysis and contributed to drafting the manuscript. J.L.R. conceived the project, designed the experiments, assisted in data analysis and contributed to drafting the manuscript.

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