

# Flock house virus replicates and expresses green fluorescent protein in mosquitoes

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Flock house virus (FHV) is a non-enveloped, positive-sense RNA virus of insect origin that belongs to the family *Nodaviridae*. FHV has been shown to overcome the kingdom barrier and to replicate in plants, insects, yeast and mammalian cells. Although of insect origin, FHV has not previously been shown to replicate in mosquitoes. We have tested FHV replication *in vitro* in C6/36 cells (derived from neonatal *Aedes albopictus*) and *in vivo* in four different genera of mosquitoes, *Aedes*, *Culex*, *Anopheles* and *Armigeres*. FHV replicated to high titres in C6/36 cells that had been subcloned to support maximum growth of FHV. When adult mosquitoes were orally fed or injected with the virus, FHV antigen was detected in various tissues and infectious virus was recovered. Vectors developed from an infectious cDNA clone of a defective-interfering RNA, derived from FHV genomic RNA2, expressed green fluorescent protein in *Drosophila* cells and adult mosquitoes. This demonstrates the potential of FHV-based vectors for expression of foreign genes in mosquitoes and possibly other insects.

## INTRODUCTION

Flock house virus (FHV), a positive-strand RNA virus of coleopteran insect origin (for reviews, see Ball & Johnson, 1998; Hendry, 1991), was originally isolated from the grass grub *Costelytra zealandica* (Coleoptera) in New Zealand and is a member of the insect virus family *Nodaviridae* (Ball *et al.*, 2000). FHV is a unique example of a virus that crosses the kingdom barrier and multiplies in plants (Selling *et al.*, 1990; Dasgupta *et al.*, 2001) and yeast (Price *et al.*, 1996). The range of insect hosts for FHV is not well studied. FHV replicates in the larvae of the wax moth, *Galleria mellonella*, and in cultured *Drosophila melanogaster* cells (Scotti *et al.*, 1983) in the laboratory. FHV has not shown any cytopathic effect (CPE) in mammalian cells and is not considered a human pathogen.

The only member of the *Nodaviridae* family shown to infect mosquitoes is nodamura virus (NOV; Ball & Johnson, 1998; Hendry, 1991). NOV infects cell lines obtained from two mosquito species, *Aedes albopictus* and *Ae. aegypti*. No CPE is observed but infectious virus is produced at a low level (Bailey *et al.*, 1975). No data are available on virus titres in these species because a plaque assay is not available for NOV. However, NOV is lethal to honey bees (Bailey & Scott, 1973) and suckling mice (Scherer & Hurlbut, 1967) and, unlike FHV, is considered pathogenic to animals.

FHV has the smallest genome of any positive-strand animal RNA virus and has been studied extensively at the molecular level. The genome of FHV consists of two RNAs packaged in

a single, non-enveloped, icosahedral virion. RNA1 (3·1 kb) encodes the viral polymerase (protein A; 112 kDa) and RNA2 (1·4 kb) encodes the viral capsid protein precursor (43 kDa). RNA1 is capable of independent replication but the replication of RNA2 is RNA1-dependent (Gallagher *et al.*, 1983). Formation of progeny virus particles requires co-infection of cells with both RNAs.

At its 3' end, FHV RNA1 encodes a subgenomic RNA3 (0·4 kb) containing two overlapping open reading frames (ORFs) encoding proteins B1 and B2. The translation of B1 is inefficient and B1 is not required for the FHV life cycle. Protein B2, translated from the second AUG in RNA3, accumulates at a high level (5% of total cell protein) in *Drosophila* cells at early stages of infection and is not packaged into the virus particle (Friesen & Rueckert, 1982). Protein B2 is not required for RNA1 replication or RNA3 synthesis (Ball, 1995); it was recently shown to suppress RNA silencing in *Drosophila* cells and in transgenic plants (Li *et al.*, 2002). Autonomous replication of FHV RNA1 and its robust capacity to synthesize viral RNAs and subgenomic RNA3 provides a promising approach towards amplifying heterologous sequences in insect cells expressed from vectors. Price *et al.* (2000) developed a yeast DNA plasmid system expressing wild-type FHV RNA1 *in vivo* and used this system to express green fluorescent protein (GFP) in yeast.

The major protein produced during FHV infection is capsid protein precursor alpha, translated from RNA2. Analysis of deletion mutants has revealed *cis*-acting sequences required

for the replication of FHV RNA2 (Ball & Li, 1993) and has shown that much of the sequence encoding the capsid protein can be deleted without severely affecting subsequent virus replication (Zhong, 1993). A defective interfering (DI) RNA (DI 634) produced after high multiplicity infection in *Drosophila* cells is a deletion mutant of FHV RNA2 containing 634 bases out of 1400 bases of RNA2. DI 634 accumulates to higher levels in infected tissues than genomic RNAs (R. Dasgupta & R. R. Rueckert, unpublished observation). Vectors derived from the cDNA clones of both FHV RNA2 and DI 634 have been used to express reporter genes such as CAT and GFP in *Drosophila* cells and yeast (Zhong *et al.*, 1992; Price *et al.*, 2000, 2002).

We wanted to explore the possibility of developing FHV-based vectors for transient gene expression and gene silencing in mosquitoes. Stable transformation of insects based on transposable elements, such as *Mariner*, *Hermes* and *piggybac*, remains a laborious procedure (see review by Atkinson *et al.*, 2001). Transient expression systems, on the other hand, are a faster and more efficient means of studying gene expression as well as gene silencing. Sindbis (SIN) virus vectors have been the most widely used viruses for gene expression in mosquitoes (Higgs *et al.*, 1999; Olson *et al.*, 1998, 2000). However, certain limitations exist for the SIN virus system: (i) SIN virus does not disseminate and replicate in all mosquito tissues; (ii) recombinant SIN virus has a size limitation of about 1 kb for inserting a gene of interest; and (iii) it is a human pathogen. Therefore, the development of other viral expression systems that are non-pathogenic to humans and preferably with a broader insect host range will be beneficial and will increase the repertoire of transducing virus expression systems. FHV-based vectors can provide both of these advantages, and the bipartite genome of FHV, which carries out distinct functions, could be engineered to express two different genes simultaneously.

Here we report that FHV replicates in a mosquito cell culture as well as in four species of mosquito. The mosquitoes tested for FHV growth are vectors of parasites (i.e. *Anopheles gambiae*, *Culex pipiens pipiens*) and viruses (i.e. *Ae. aegypti*, *C. pipiens pipiens*) that have a significant impact on global public health, or are model organisms for studies of mosquito immune responses (i.e. *Armigeres subalbatus*). We have also demonstrated that a vector derived from a cDNA clone of FHV DI RNA is capable of expressing GFP in mosquito cells and tissues.

## METHODS

**Maintenance of mosquitoes.** Mosquitoes used in this study were *Ae. aegypti* (black-eye Liverpool strain), *C. pipiens pipiens* (Iowa State strain), *A. gambiae* (Plasmodium-susceptible strain 4arr) and *A. subalbatus*. Mosquitoes were reared according to described methods (Christensen & Sutherland, 1984; Gerberg *et al.*, 1994).

**Cells, propagation of virus and extraction of virion RNA.** *D. melanogaster* cells (DL1) were grown at 26 °C in Schneider's insect cell medium (Schneider, 1972) supplemented with 10% foetal bovine serum (FBS). C6/36 cells (derived from *Ae. albopictus*; ATCC

CCL-126) were maintained in Leibovitz L-15 medium containing 10% FBS and were grown at 28 °C in close-capped tissue culture flasks. FHV was propagated in DL1 cells, purified by sucrose gradient centrifugation and virus titre was determined by plaque assay on *Drosophila* cell monolayers (Selling & Rueckert, 1984). DL1 cells infected with FHV at an m.o.i. of 0.1–1 for 48 h were the source of virus for blood feeding and injecting mosquitoes. FHV RNA isolated from purified virus by phenol extraction was used for liposome-mediated transfection of insect cells.

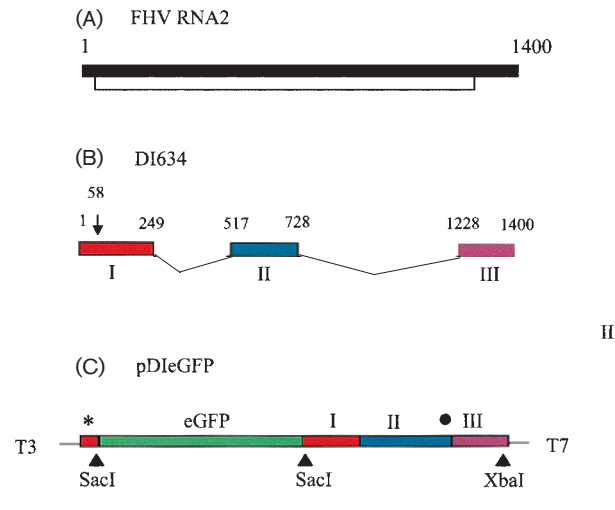
**Growth of FHV in C6/36 mosquito cells.** C6/36 cells were passed the day before inoculation and allowed to reach 85–90% confluency before inoculation with FHV at an m.o.i. of 1 (inocula were approximately  $1.7 \times 10^6$  p.f.u.). FHV was obtained from the supernatant of virus-infected *Drosophila* cells. After 1 h incubation at 28 °C, the inoculum was removed and cells were washed twice with fresh medium, then incubated at 28 °C for up to 96 h. Cell supernatant was collected at 24 h intervals post-infection after two rounds of freeze-thawing to release intracellular viruses and centrifugation to remove cell debris. Virus titre was determined by plaque assay on *Drosophila* cells. Inoculated C6/36 cells were also subjected to an immunofluorescence assay (IFA).

In order to clone the subsets of C6/36 cells that support optimum growth of FHV, C6/36 cells were serially diluted and seeded on a six-well plate to obtain a single-cell suspension; cells were then overlaid with medium containing agar. Individual colonies were isolated after 7–10 days, transferred to microtitre wells and allowed to grow for an additional 3 days. Cells from individual clones were inoculated with FHV at an m.o.i. of 1, fixed on glass slides after 72 h and FHV growth was monitored by IFA. Cells that promoted enhanced growth were further subcloned to obtain a clonal cell line that supported maximum growth of FHV.

**Infection of mosquitoes with FHV.** Supernatants from FHV-infected *Drosophila* cells were used to infect mosquitoes by injection (Beernsten & Christensen, 1990). Individual mosquitoes were injected with 0.5–1 µl containing approximately  $5 \times 10^4$ – $5 \times 10^5$  p.f.u. infectious virus. The same source of FHV was used to infect mosquitoes per os. Mosquitoes were exposed to FHV mixed with an artificial blood meal (Kogan, 1990) at a 1:1 ratio (virus titres were  $1$ – $2 \times 10^8$  p.f.u. ml<sup>-1</sup>) through a Parafilm membrane or chicken skin on a water-jacketed membrane feeder (Rutledge *et al.*, 1964).

**Detection of FHV growth.** FHV growth was monitored in both cell cultures and mosquitoes using an IFA and a plaque assay on *Drosophila* cells. Control and virus-exposed mosquito tissues were removed by dissection and assayed for the growth of virus by IFA (Cheng *et al.*, 2001). Polyclonal anti-FHV antibody (raised against capsid protein) and FITC-labelled goat anti-rabbit IgG at dilutions of 1:400 and 1:800, respectively, were used as primary and secondary antibodies for virus detection. The fluorescence was examined in cells or tissues with UV illumination using an Olympus IX70 inverted microscope. For plaque assays, five virus-exposed mosquitoes from each species were homogenized with a plastic pestle in 1.5 ml Eppendorf tubes in isotonic buffer (IB; 100 mM NaCl, 35 mM Pipes, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, pH 6.8). Supernatant was collected after centrifugation and plaque assays were performed after diluting  $10^4$ – $10^5$  in IB. The resultant titre was divided by five to determine approximate p.f.u. per mosquito.

**Plasmid constructs for expression of GFP.** cDNA clones of FHV DI RNA in pBluescript (pDI634; Zhong *et al.*, 1992; Zhong, 1993) and pEGFP (Clontech) were used to construct pDIeGFP (Fig. 1C). The eGFP ORF (nt 289–1105) was amplified from pEGFP by PCR using the eGFP-specific primers 5'-GTCGAGCTCAAATG GTGAGCAAGGGCG-3' and 5'-GTCGAGCTCTCTTGTACAGCTC GTCC-3', both containing *SacI* sites at their 5' ends. The PCR



**Fig. 1.** Structure of the plasmid pDIeGFP. Schematic drawing of FHV genomic RNA2 (A) and defective-interfering RNA DI 634 (B). The solid bar represents RNA2 sequences and the open box represents the open reading frame for the FHV capsid protein precursor. The three regions of RNA2 (nt 1–249, 517–728 and 1228–1400 designated I, II and III, respectively) retained in DI 634 are shown in different colours. Deleted sequences are indicated by thin lines. (C) Linear map of the pDIeGFP construct showing the DI (I, II and III) and eGFP (green) sequences and the promoters for transcription on either side. Location of the restriction sites used for eGFP insertion (*SacI* at nt 58 of DI634; indicated by an arrow in Fig. 1B) and linearization (*XbaI* at nt 1360) of the plasmid are indicated. The asterisk and solid circle show the locations of the start codon (nt 23) and the termination codon (nt 1225) for the DI–eGFP fusion protein, respectively.

**Table 1.** Comparison of FHV titres in original and cloned C6/36 cell lines

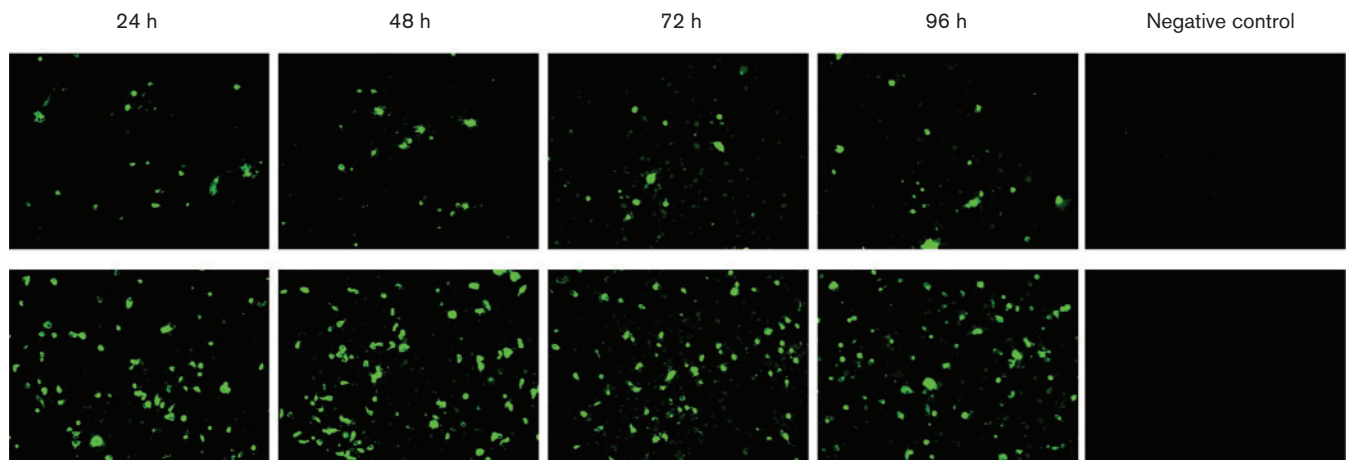
C6/36 cells	FHV titre (p.f.u. ml <sup>-1</sup> )			
	24 h	48 h	72 h	96 h
Non-cloned	1.1 × 10 <sup>6</sup>	3.1 × 10 <sup>6</sup>	5.7 × 10 <sup>6</sup>	7.1 × 10 <sup>6</sup>
Clone 8-12-8	1.4 × 10 <sup>8</sup>	3.9 × 10 <sup>8</sup>	1.3 × 10 <sup>8</sup>	3.7 × 10 <sup>8</sup>

product was then transferred to the *SacI* site at nt 58 of FHV RNA2 (Dasgupta & Sgro, 1989; GenBank accession no. X15959) using standard techniques (Sambrook *et al.*, 1989). FHV DI RNA contained nt 1–249, 517–728 and 1228–1400 of RNA2 (Fig. 1A, B) and contained all the signals for replication and encapsidation (Zhong *et al.*, 1992).

**In vitro transcription and transfection.** Capped mRNA transcripts were generated using Ampliscribe kits (Epicenter Technologies). pDIeGFP was linearized with *XbaI* and transcribed with T3 RNA polymerase. The RNA transcripts were used for transfection without further purification. Residual DNA template was not removed by DNase treatment.

*Drosophila* or C6/36 cells, at 50–70% confluency in a six-well plate, were transfected with 1–3 µg RNA transcripts using the TransMessenger transfection reagent (Qiagen) according to the manufacturer's protocol. Approximately 0.5 µg purified FHV RNA was used for co-transfection in each experiment. Cells were monitored for GFP expression up to 96 h post-transfection. Supernatant and cells were subjected to two cycles of freeze-thawing to release virus particles from transfected cells. Green fluorescence was monitored in cells after 48–72 h by UV illumination using an Olympus IX70 inverted microscope and images were captured by a Spot RT slider digital camera (Diagnostic Instruments, Inc.).

**In vivo expression of GFP in mosquitoes.** FHV DIeGFP virus was generated from *Drosophila* cells co-transfected *in vitro* with pDIeGFP transcripts and FHV genomic RNAs. Virus was harvested from cells at 72 h post-transfection after two cycles of freeze-thawing



**Fig. 2.** FHV growth in original (top) and subcloned (bottom) C6/36 cells at different times post-infection. Clone #8-12-8 showed maximum number of infected cells at 48–72 h post-infection as detected by an immunofluorescence assay. Uninfected cells (negative controls) showed no fluorescence.

**Table 2.** FHV titres in four different mosquito species injected intrathoracically

Species	FHV titre (p.f.u. per mosquito)			
	3 days p.i.	5 days p.i.	7 days p.i.	10 days p.i.
<i>Culex pipiens pipiens</i>	$1.8 \times 10^4$	$2.4 \times 10^4$	$4.3 \times 10^5$	$3.6 \times 10^5$
<i>Aedes aegypti</i>	$2.0 \times 10^4$	$8.2 \times 10^4$	$1.4 \times 10^6$	$1.9 \times 10^6$
<i>Armigeres subalbatus</i>	$2.7 \times 10^4$	$4.8 \times 10^4$	$4.6 \times 10^5$	$4.1 \times 10^5$
<i>Anopheles gambiae</i>	$1.4 \times 10^4$	$9.6 \times 10^5$	$1.8 \times 10^6$	$1.2 \times 10^6$

and subjected to centrifugation at 8000 r.p.m. for 10 min to remove cell debris. The supernatant was inoculated into *Ae. aegypti* either directly or after concentration by ultracentrifugation. To concentrate the FHVΔIEGFP virus, supernatant from transfected cells was first pelleted over a 30% sucrose solution and resuspended in 4 ml STE buffer (10 mM Tris/HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5). Virus was pelleted again by ultracentrifugation and resuspended in 40 µl STE resulting in an increase in virus concentration of 100-fold. Approximately 0.5 µl of the resuspended virus was injected into individual *Ae. aegypti* mosquitoes. Mosquitoes were monitored at 3, 7, 10 and 14 days post-injection and various tissues were dissected and examined for GFP expression.

## RESULTS AND DISCUSSION

### FHV replicates in mosquito cells

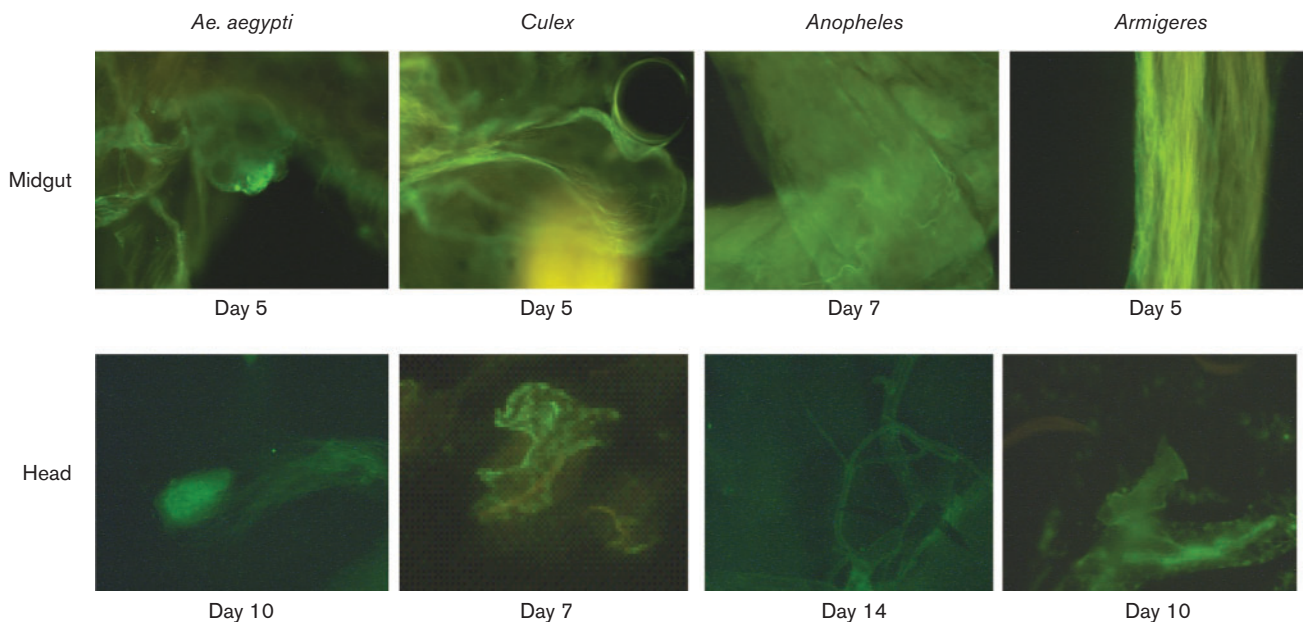
Maximum growth of virus in stock C6/36 cell line was detected 72–96 h post-infection; however, certain subsets of

cells were not infected, even when the amount of FHV inoculum and the incubation time were increased (data not shown). This was not surprising since the C6/36 cell line was derived from neonatal tissues of *Ae. albopictus* and is probably heterogeneous.

Three rounds of subcloning produced a C6/36 clone #8-12-8 that showed a dramatic increase (approximately 100-fold) in the ability to amplify FHV compared with the original cells, as measured by virus titration (Table 1). IFA (Fig. 2) with anti-FHV antibodies also confirmed that more C6/36 cells in the cloned cell line #8-12-8 were infected. The growth of original C6/36 cells versus the cloned cells showed no considerable differences: the cell doubling time was roughly the same (24–36 h) and the fluorescence intensity of individual infected cells was similar between the original and cloned cell line. Collectively, these data indicated that FHV replicated equally well in infected cells from both cell lines but that the cloned cell line had more cells susceptible to FHV infection. No CPE was detected in the FHV-infected C6/36 cells. This clonal cell population, selected for susceptibility to FHV infection, could be valuable for studies of physiological processes in mosquito cell lines.

### FHV replicates in different genera of mosquitoes

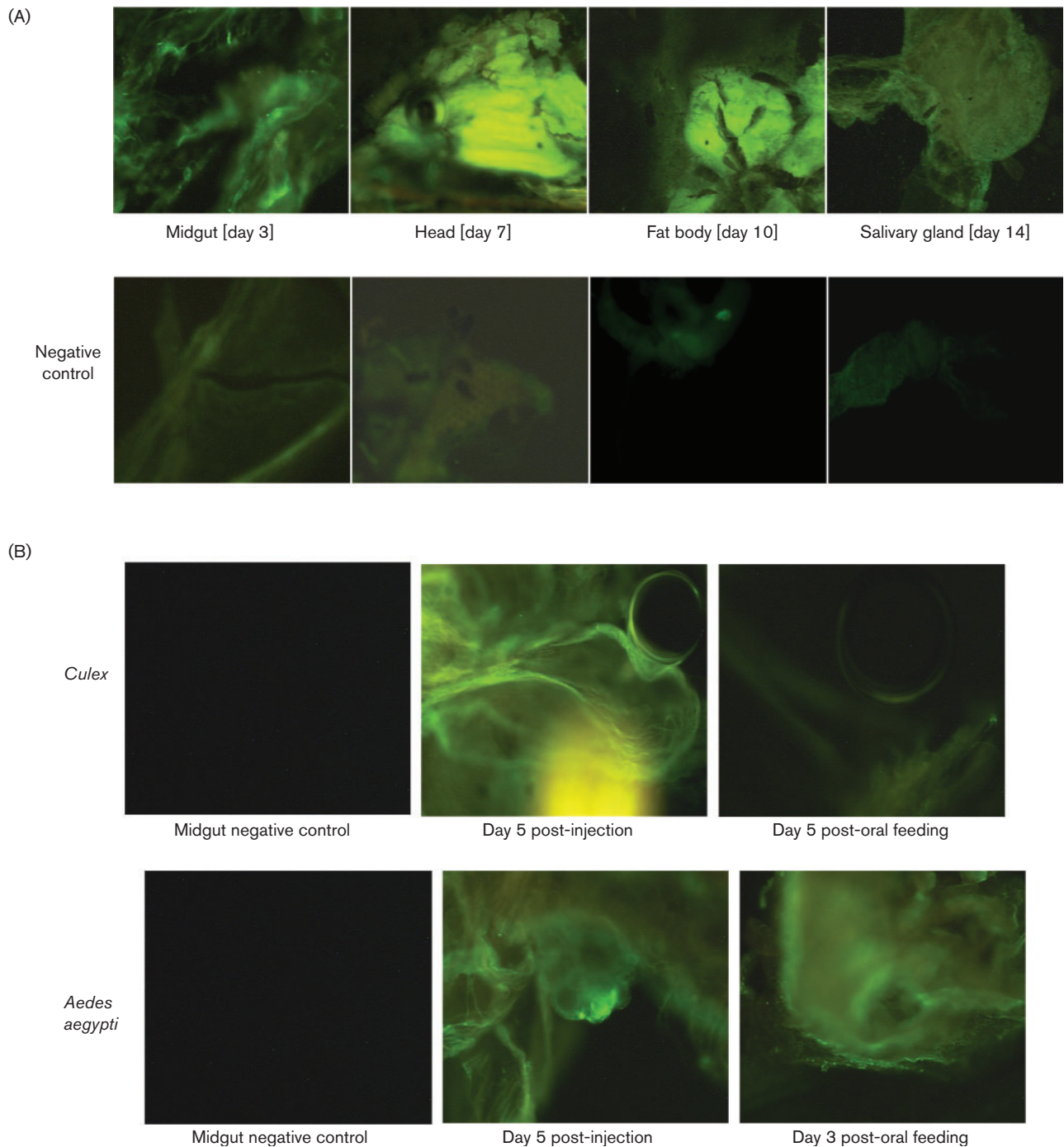
FHV growth in four species of mosquito was monitored at 3–14 days post-inoculation (p.i.) by plaque assay of the infected mosquito homogenates on *Drosophila* monolayers. An increase in the virus titre with time (5–10 days p.i.) following exposure was observed in all four species injected with FHV (Table 2). The titres were obtained from



**Fig. 3.** FHV growth detected by an immunofluorescence assay in the tissues of four different species of mosquito injected intrathoracically with virus. Number of days post-inoculation and dissected tissues are indicated.

homogenates of five mosquitoes injected with FHV. Overall, FHV titre increased about 25-fold in *Culex* and *Armigeres* and about 100-fold in *Aedes* and *Anopheles*. The initial drop in the virus titre at 3 days p.i. compared with the inoculum ( $5 \times 10^4$ – $10^5$  p.f.u.) could have been due to variation in the uptake of virus and/or inactivation of some FHV by

different mosquitoes. Virus titres in orally fed mosquitoes also increased with time but varied due to variations in feeding of different mosquito species (data not shown). Many *Culex* and *Armigeres* mosquitoes did not feed to repletion, resulting in a lower infectious dose of FHV. However, mosquitoes that fed well showed an increase in



**Fig. 4.** FHV growth detected by an immunofluorescence assay in different tissues of *Ae. aegypti* (A) and midgut tissues of *Ae. aegypti* (LVP) and *C. pipiens pipiens* (B) orally fed with FHV. Method of exposure, number of days post-infection and the type of tissue are indicated.

virus titre comparable with the values of those that were injected. None of the FHV-infected mosquitoes tested showed signs of adverse effects or behavioural changes related to virus infection.

We monitored the growth of FHV in the midgut and head tissues of the four mosquito species by IFA. FHV growth was evident in the midgut (5–7 days p.i.) and head tissues (7–14 days p.i.) of all four species when injected intrathoracically (Fig. 3). Virus titres in these tissues were not measured due to possible contamination from haemolymph and other organs during dissection. Tissues from non-inoculated mosquitoes showed no fluorescence in IFAs (data not shown).

### Growth of FHV in different mosquito tissues

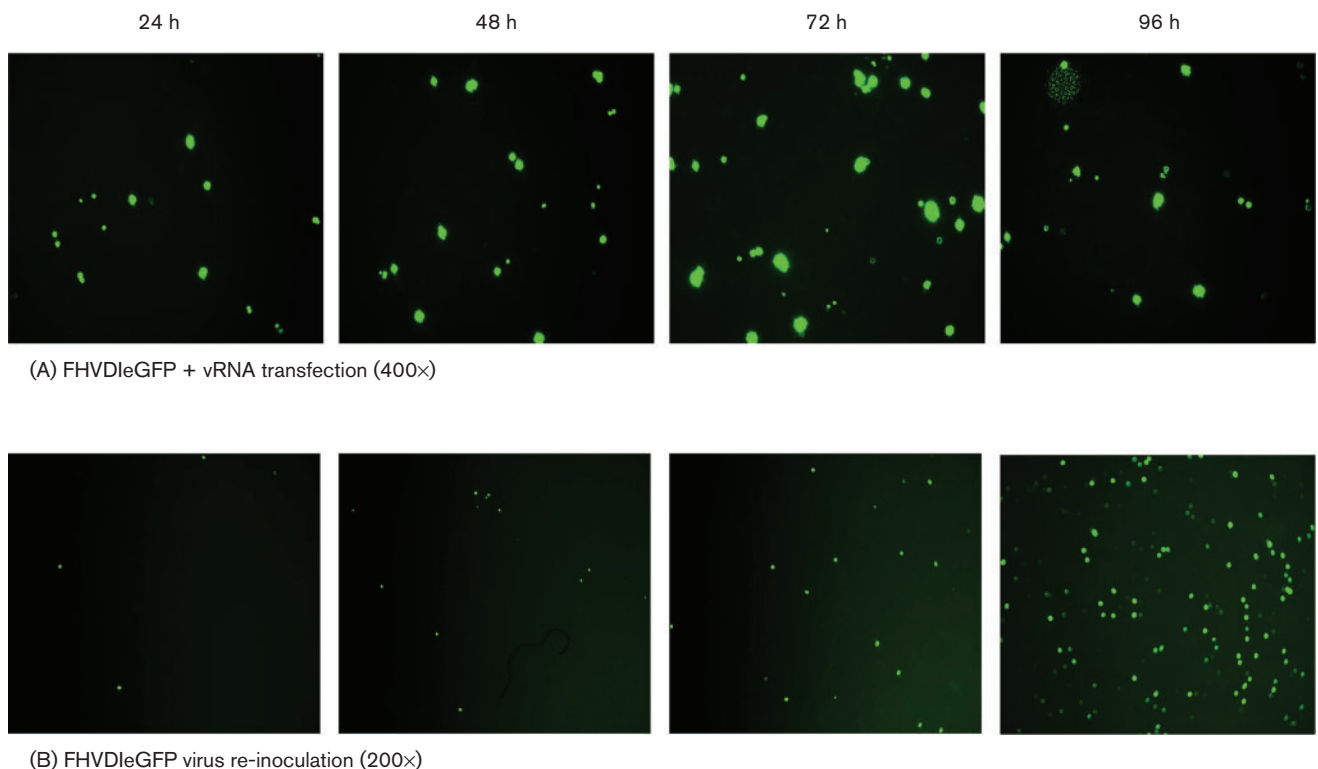
We specifically assessed virus growth in tissues involved in mosquito–pathogen interactions. The midgut functions as the first barrier that a pathogen encounters when taken up with a blood meal. The fat body is responsible for the secretion of immune molecules, as well as synthesis of yolk proteins. Thoracic muscles serve as the site of development for various filarial worms. Head tissues and salivary glands in particular serve as barriers to parasite and arbovirus transmission (for review, see Beerntsen *et al.*, 2000). FHV replicated in all of these tissues except thoracic muscles

following blood feeding or inoculation with the virus (Fig. 4).

Growth and dissemination of FHV was examined in orally fed *Ae. aegypti* in greater detail. IFA images showed the presence of FHV in the midgut epithelial cells, head, fat body and salivary glands (Fig. 4A) of orally fed *Ae. aegypti* at 3–14 days p.i. The earliest appearance of FHV was in midgut (3 days p.i.) followed by dissemination to the fat body, head (7–10 days p.i.) and salivary gland (14 days p.i.). Both *Ae. aegypti* and *C. pipiens pipiens* exposed to FHV by oral feeding or intrathoracic inoculation showed the presence of virus in the fat body, head tissues and tracheae surrounding the midgut. Fig. 4(B) shows FHV growth in the midgut tissues of these two species 5 days p.i. by either method. Weaker fluorescence in orally fed *Culex* (5 days p.i.) compared with *Aedes* (3 days p.i.) midgut was probably due to the poor intake of virus from the smaller blood meal taken by *Culex*.

### FHV vectors express the reporter gene GFP *in vitro* in *Drosophila* cells and mosquito cells

We inserted the sequence of eGFP near the 5' end (nt 58) of the DI RNA so that eGFP was efficiently translated using the ATG (nt 23) and leader sequence of the FHV capsid protein (Fig. 1). The transcript made from linearized pDIeGFP was



**Fig. 5.** GFP expression in *Drosophila* cells using the FHV-based vector DIeGFP. (A) FHVDIeGFP RNA transcripts were co-transfected into *Drosophila* cells with FHV RNA extracted from sucrose gradient-purified virus. Images were captured at times post-transfection as indicated. (B) FHVDIeGFP viruses, isolated 72 h after the transfection in (A), were re-inoculated into *Drosophila* cells and monitored for GFP expression up to 96 h.

1364 bases long and contained an ORF of 1203 bases. The resulting fusion protein (401 aa) contained the first 13 aa of the FHV capsid protein followed by eGFP (239 aa), part of the FHV capsid protein (75 aa coded by nt 24–249 of the DI RNA) and a random sequence of 74 aa at the C terminus.

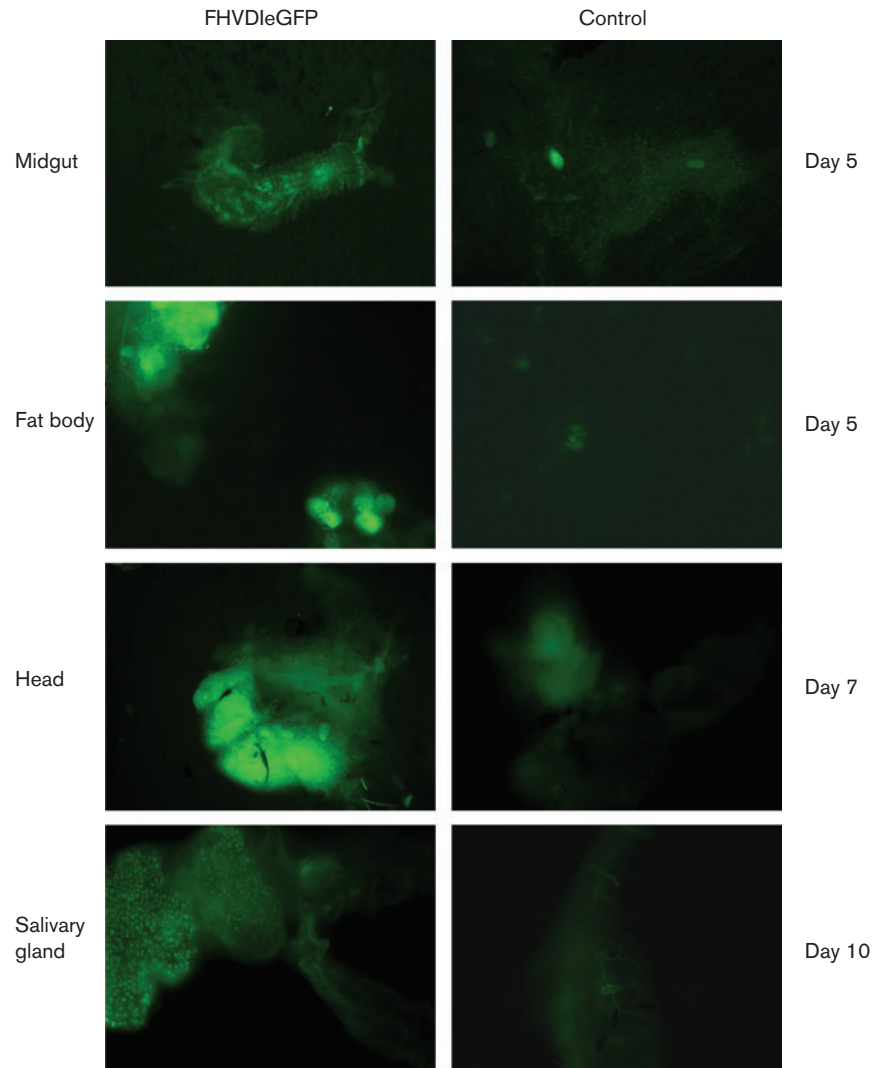
GFP expression was evident in *Drosophila* cells that were co-transfected with this transcript and FHV virion RNAs. We observed a high level of GFP expression in *Drosophila* cells, which increased with time and peaked at 72 h post-transfection (Fig. 5A). We reinfected fresh cells with supernatants from these transfected cell lysates and detected FHV and GFP expression at different time points (Fig. 5B) confirming that virus particles were made in *Drosophila* cells and GFP sequences were retained in the progeny viruses.

In contrast to *Drosophila* cells, C6/36 cells could not be efficiently transfected using similar conditions and showed

poor expression of GFP. However, C6/36 cells infected with supernatant from *Drosophila* cell lysates from both passages (FHV-transfected or reinfected cells) showed GFP fluorescence (data not shown).

### FHV vectors express the reporter gene GFP *in vivo* in *Ae. aegypti*

When supernatant from *Drosophila* cells transfected with transcripts derived from pDIeGFP was used to infect mosquitoes by injection in an *in vivo* experiment, GFP expression was detected in five mosquitoes out of 40 examined (data not shown). In contrast, when supernatants were concentrated by ultracentrifugation and injected into mosquitoes, GFP expression was detected in 17 out of 50 mosquitoes tested. Of the 17 mosquitoes, GFP fluorescence was observed in the head tissue of 12, the fat body of nine, the



**Fig. 6.** GFP expression *in vivo* in *Ae. aegypti* LVP strain injected with FHVDIeGFP virus. Virus was recovered from transfected *Drosophila* cells (see Fig. 5). Expression was measured in various tissues at different days post-inoculation as indicated.

midgut of seven and the salivary glands of five mosquitoes. A representative collection of these images is shown in Fig. 6.

These results indicate that infectious DIeGFP FHV particles made in *Drosophila* cells were infectious but not infectious enough to infect 100% of injected mosquitoes. This could be a result of the small volume used to inject each mosquito. In addition, when DI RNA transcripts are co-transfected with FHV genomic RNAs, three types of virus particle could be generated: (i) genomic RNA1 and RNA2 could generate infectious virus particles and assemble together with one copy of the DI RNA/GFP sequence; (ii) genomic RNA1 could replicate both RNA2 and DI/eGFP sequences resulting in virus particles with one copy of genomic RNA1 and one or two copies of DI RNA/GFP (no RNA2 is encapsidated); and (iii) virus particles containing multiple copies of DI RNA/GFP could be assembled (no genomic RNA encapsidated). In the latter two cases, the particles would not be able to generate infectious progeny virus because genomic RNA2 is not encapsidated. Our results showing GFP expression confirmed that some FHV particles must have assembled via pathway (i) and encapsidated the eGFP sequence in addition to genomic RNAs. By concentrating the virus, more infectious FHV DIeGFP virus was injected into individual mosquitoes resulting in more mosquitoes expressing GFP (Fig. 6). Further development and optimization of pDIeGFP and FHV genomic RNA-based vectors are in progress in order to improve GFP expression.

Our data show that FHV is capable of replicating both in cultured mosquito cells and *in vivo* in mosquito tissues including the midgut, head tissue, fat body and salivary glands without signs of pathogenicity. In addition, the replication machinery of FHV can be used to express foreign sequences in mosquitoes. Our findings extend the host range of FHV to another order of insects (Diptera) and raise the possibility that FHV will replicate in various insects. Preliminary studies have demonstrated that FHV multiplies to high titres in tsetse flies (*Glossina moritans*) and expresses viral antigen in various tissues (data not shown). These data, in addition to the small size and wide host range of FHV, suggest that FHV-based vectors could prove extremely useful for studying insect molecular biology, gene expression (with the possibility of expressing two genes simultaneously) and gene silencing.

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