Characterization of an endogenous gene expressed in Aedes aegypti using an orally infectious recombinant Sindbis virus

Authors: Cheng, L. L., Bartholomay, L. C., Olson, K. E., Lowenberger, C., Vizioli, J., et al.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Characterization of an endogenous gene expressed in *Aedes aegypti* using an orally infectious recombinant Sindbis virus

L.L. Cheng[1], L.C. Bartholomay[1], K.E. Olson[2], C. Lowenberger[1], J. Vizioli[3], S. Higgs[2], B.J. Beaty[2] and B.M. Christensen[1]

[1]*AHABS, University of Wisconsin, Madison, WI, USA; AIDL,* [2]*Colorado State University, Fort Collins, CO, USA;* [3]*Institut de Biologie Moleculaire et Cellulaire, Strasbourg, France*
cheng@ahabs.wisc.edu

Abstract

Sindbis virus expression vectors have been used successfully to express and silence genes of interest *in vivo* in several mosquito species, including *Aedes aegypti*, *Ae. albopictus*, *Ae. triseriatus*, *Culex pipiens*, *Armigeres subalbatus* and *Anopheles gambiae*. Here we describe the expression of an endogenous gene, defensin, in *Ae. aegypti* using the orally infectious Sindbis virus, MRE/3'2J expression vector. We optimized conditions to infect mosquito larvae *per os using C6/36 Ae. albopictu*s cells infected with the recombinant virus to maximize virus infection and expression of defensin. Infection with the parental Sindbis virus (MRE/3'2J) did not induce defensin expression. Mosquito larvae infected by ingestion of recombinant Sindbis virus-infected C6/36 cells expressed defensin when they emerged as adults. Defensin expression was observed by western analysis or indirect fluorescent assay in all developmental stages of mosquitoes infected with MRE/3'2J virus that contained the defensin insert. The multiplicity of infection of C6/36 cells and the quantity of infected cells consumed by larvae played an important role in defensin expression. Parental viruses, missing the defensin insert, and/or other defective interfering virus may have contributed to these observations.

Keywords: Sindbis virus, defensin, gene expression, MRE/3'2J, mosquito

Abbreviation:

BHK cells	baby hamster kidney cells
dsSIN	SIN containing a second subgenomic promoter
IFA	indirect fluorescence assay
MAb	Monoclonal antibody
MOI	Multiplicity of infection (number of infectious virus particles inoculated per cell)
ppA	preprodefensin A
ppC	preprodefensin C
SIN	Sindbis virus
$TCID_{50}$	Tissue culture infections dose 50% end point

Introduction

Sindbis (SIN) virus is a positive sense single-stranded enveloped RNA virus (*Togaviridae* family) that naturally cycles between mosquitoes and avian hosts (Taylor *et al.*, 1955). Recombinant Sindbis viruses have been used to express or silence genes of interest both *in vitro* and *in vivo* and offer great potential for gene characterization (Jiang *et al*, 1995, Gaines *et al.*, 1996, Higgs *et al.*, 1996, Powers *et al.*, 1996, Kamrud *et al.*, 1997, Johnson *et al.*, 1999, De Lara Capurro *et al.*, 2000, Shiao *et al.*, 2001). The double subgenomic SIN (dsSIN) virus systems contain a second subgenomic promoter between the structural protein genes and the non-coding region to facilitate the expression of inserted genes (Hahn *et al.*, 1992, Olson *et al.*, 1994, 2000). The utility of the dsSIN virus expression system has been demonstrated in a number of studies; heterologous genes have been expressed both *in vitro* and *in vivo* (Higgs *et al.*, 1996, Kamrud *et al.*, 1997, Olson *et al.*, 2000), bunyavirus and flavivirus replication and transmission were blocked (Gaines *et al.*, 1996, Jiang *et al*., 1995, Olson *et al.*, 1996, Powers *et al.*, 1996, Adelman *et al.*, 2001), a specific gene was silenced to demonstrate its importance in a biosynthetic pathway (Shiao *et al.*, 2001), and *Plasmodium gallinaceum* sporozoites were unable to

infect salivary glands of *Ae. aegypti* infected with a dsSIN virus expressing single chain antibody to circumsporozoite protein (De Lara Capurro *et al.*, 2000). Here we describe the use of an orally infectious SIN virus, MRE/3'2J, to express an endogenous *Ae. aegypti* gene involved in the antimicrobial immune response.

Insects produce an array of potent antimicrobial peptides in response to bacterial invasion (Hoffmann *et al.*, 1999). Activation of this inducible, innate immune response in *Ae. aegypti* results in the reduction of establishment of the eukaryotic parasites *P. gallinaceum* and *Brugia malayi* (Lowenberger *et al.*, 1996, 1999a). In order to evaluate specific immune peptides potentially involved in this observed anti-parasitic effect, we engineered the orally infectious dsSIN virus, MRE/3'2J to express *Ae. aegypti* defensin genes A and C (Lowenberger *et al.*, 1999b). These genes are expressed in a tissue specific manner following bacteria inoculation, and are not induced by blood feeding (Lowenberger *et al.*, 1996, 1999a). Defensin A is produced mainly in the fat body and released into the hemolymph, and defensin C is produced primarily in the midgut (Lowenberger *et al.*, 1999a). The orally infectious MRE/3'2J virus was used in order to circumvent the possibility that other genes involved in wound healing or the innate immune response might be induced if the dsSIN TE/3'2J (Higgs *et al.*, 1996) system, which requires inoculation, was used instead.

In this study, we evaluate different means of infecting mosquitoes with recombinant SIN viruses in order to optimize the prevalence of infection and defensin expression, demonstrate that mosquitoes exposed to SIN virus-infected C6/36 *Ae. albopictus* cells as larvae show different levels of infection (% of infected mosquitoes/total number assayed) and defensin expression levels, as compared to adult mosquitoes exposed to these viruses via an infected blood meal, and show that both the multiplicity of infection (MOI) in C6/36 cells and the amount of virus to which larvae are exposed play critical roles in the pattern of defensin expression in infected mosquitoes.

Materials and Methods

Virus Production

Development of the orally infectious recombinant SIN virus MRE/3'2J and chimeric SIN viruses expressing reporter genes has been described previously (Higgs *et al.*, 1999, Olson *et al.*, 2000, Seabaugh *et al.*, 1998). MRE/3'2J plasmids containing inserts of preprodefensin A (ppA) or preprodefensin C (ppC) (Lowenberger *et al.*, 1995, Lowenberger *et al.*, 1999b) were prepared from *Escherichia coli* (DH?5 strain) bacterial cells grown overnight in Terrific broth (Sambrook *et al.* 1989). DNA was isolated using the QIAfilter Midi Kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was linearized by restriction digest with 3-4 fold excess of Xho I. Complete digestion of the DNA was confirmed by agarose gel electrophoresis. DNA was transcribed *in vitro* from the SP6 promoter, and RNA capping was achieved by adding a capping analog (Ambion, Inc.) to the mixture. This RNA was electroporated into 5×10^6 BHK-21 (baby hamster kidney) cells using a BioRad Gene Pulser set at 450 V, 125 µF, for 0.9 s. Cells and debris from the electroporation were immediately added to 4 ml of Leibovitz L-15 medium (Gibco BRL) supplemented with 10% fetal bovine serum in 25 cm^2 tissue culture flasks. Viruses were

harvested from BHK cells and titrated (plaque forming unit and tissue culture infectious dose 50% end-points (TCID$_{50}$)) 24 hours after transfection. Approximately $5\text{-}8 \times 10^7$ plaque forming units or 7.2-8 log10 TCID$_{50}$ per ml of MRE/2'3J, MRE/2'3J/ppA, and MRE/2'3J/ppC viruses were obtained.

To confirm the recombinant viruses contained defensin inserts, purified viruses from BHK cells were used to extract viral RNA (Chandler *et al.*, 1990) and subsequently used in a reverse transcription and polymerase chain reaction (RT-PCR). A 5' primer specific for defensin genes and an oligo dT primer were used in the PCR reaction (Lowenberger *et al.*, 1999b).

Analysis of progeny in virus stock

To determine if the parental MRE/3'2J virus (without the defensin insert) was present in the viral stocks of MRE/3'2J/ppA or ppC, BHK cells were inoculated with either the original stock or another preparation obtained by re-inoculation of the original stock at a MOI of 0.01, and individual plaques of viruses were isolated. After plaque purification, samples were inoculated into C6/36 cells maintained in L-15 medium with 10% fetal bovine serum on coverslips at a MOI of 1. Infected cells were harvested 72 hours after infection and expression of defensin and SIN E1 protein were assessed by indirect fluorescence assay (IFA).

Per os *infection of mosquitoes*

Infection of *Ae. aegypti* larvae with dsSIN viruses were recently described by Higgs *et al.* (1999), and minor modifications of these procedures were employed. C6/36 mosquito cells (*Ae. albopictus* origin) were inoculated with virus at MOIs of 0.1 or 0.01 and incubated at 28ºC for 48 hours. Cells were resuspended using a cell scraper. *Ae. aegypti* Liverpool eggs were hatched in deoxygenated water and first instar larvae were immediately transferred to flasks containing virus-infected cells. Larvae were maintained at 28º C, and after infected cells were completely consumed (approximately 2 to 3 days post exposure), larvae were washed thoroughly in distilled water and transferred to normal rearing conditions (Beerntsen *et al.*, 1990).

To infect adult *Ae. aegypti*, three day old adults were exposed to infectious blood meals through a Parafilm™ membrane on a water-jacketed membrane feeder (Rutledge *et al*., 1964). To prepare the infectious blood meals, C6/36 cells were inoculated with virus at a MOI of 0.1, incubated at 28º C for 48 hours, and suspended in defibrinated sheep blood at 1:1 ratio.

Analysis of infection

Tissues from virus-exposed mosquitoes were removed by dissection and assayed for viral infection by indirect fluorescence assay (IFA) or by isolating virus in tissue culture. In order to confirm infection, dissected midguts were fixed on slides in cold acetone for 1 hour. Midguts were then examined by IFA. A monoclonal antibody (MAb) 30.11a, raised against the Sindbis E1 envelope protein, was diluted 1:200 in PBS and added to the tissue samples, which were incubated at room temperature for 1 hour and then washed with PBS 3 times. A Texas-Red conjugated secondary antibody (diluted 1:400) was added and incubated for 40 min, followed by three washes before mounting with Mowiol solution (Harlow *et al.*, 1988). Alternatively, carcasses of dissected
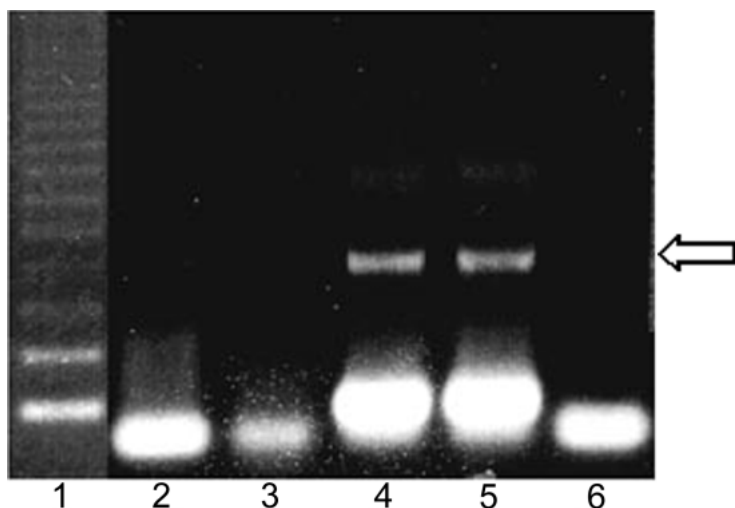
Figure 1. Detection of defensin sequences in recombinant Sindbis viruses by RT-PCR. Viruses were amplified in BHK cells. Cells and parental virus, MRE/3'2J, were used as the negative controls (lane 2 and 3, respectively), and products from MRE/2'3J/ppA (lane 4) and MRE/2'3J/ppC (lane 5) show the defensin gene (arrow). Molecular weight marker is lane 1. No RNA template was added in lane 6.

mosquitoes were ground in microcentrifuge tubes in 100 ?l of L-15 medium using pestles. Samples were centrifuged at 5220 x *g* for 10 min, the supernatant was inoculated into BHK-21 cells in culture, and cells were monitored for cytopathic effect. Alternatively, samples were titrated to determine the virus titer of infected mosquitoes.

Analysis of defensin expression

Defensin expression was monitored in hemolymph or midguts of virus-exposed mosquitoes by western analysis. Hemolymph was collected by perfusion as previously described (Beerntsen *et al.*, 1990), dried (DNA Speed Vac 110, Savant), and resuspended in denaturing loading buffer (25% 4X Tris-Cl/SDS pH 8, 20% glycerol, 1% SDS, 0.5% 2-Mercaptoethanol), and boiled for 5 min. For analysis of expression in the midgut, tissue was removed from mosquitoes into 10 µL chilled cell lysis buffer (NET buffer, 0.5% NP-40, 2 µg/µL aprotinin). Midgut samples were incubated on ice for 30 minutes, sonicated and centrifuged. The supernatant of lysed midguts was mixed with loading buffer and boiled for 3-5 min. Larvae and pupae were ground with pestles and lysed in cell lysis buffer and prepared in the same manner as the midgut tissues. Samples were subjected to electrophoresis in 18% SDS-PAGE gels at 200V using a Criterion gel system (Bio-Rad) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blotted with a polyclonal anti-defensin antibody raised against recombinant defensin in rabbit (diluted 1:45,000), followed by a horseradish peroxidase (HRP) conjugated secondary antibody (diluted 1:3000), and exposed to Lumi-Light Western Blotting Substrate (Roche) according to the manufacturer's instructions.

In some cases, virus-inoculated cell culture and midgut tissue from infected mosquitoes was subjected to IFA for defensin expression. Samples were examined by double-staining with an antibody to defensin and a MAb to the E1 Sindbis virus envelope protein. These were stained differentially with a fluoroisothiocyanate

(FITC) (for anti-defensin antibody), or a Texas Red (for anti-E1 protein MAb) conjugated secondary antibody.

Results and Discussion

Virus Production

RT-PCR analysis demonstrated that the recombinant MRE/2'3J/ppA and MRE/2'3J/ppC viruses generated from cDNA clones contained the proper defensin inserts (Figure 1). In addition, expression of defensin by MRE/3'2J/ppA or ppC was confirmed in infected C6/36 cells (Figure 2). Interestingly, even though the titers of MRE/2'3J/ppA and MRE/2'3J/ppC viruses in cell cultures were similar (8.07 ± 0.4 log $_{10}$ TCID$_{50}$/ml for MRE/2'3J/ppA and 8.0 ± 0.3 log $_{10}$ TCID$_{50}$/ml for MRE/2'3J/ppC 48 hours after infection), defensin A peptides were initially detected almost 24 hours earlier in the cell culture after viral inoculation, and in greater abundance than defensin C peptides (Figure 2).

Virus Dissemination

Midgut, head, thorax, Malpighian tubules and reproductive tissues were dissected and examined for the dsSIN virus dissemination. The tissue tropism of the MRE/3'2J recombinant viruses had been determined previously in mosquitoes infected as adults or as larvae (Seabaugh *et al.*, 1998, Higgs *et al.*, 1999, Olson *et al.*, 2000). Sindbis virus E1 glycoprotein and defensin were detected primarily in midgut tissue from infected mosquitoes (Figure 3), which is an ideal location for expression of genes that may affect parasites taken up with a blood meal. Defensin was not detected in midguts of mosquitoes infected with MRE/3'2J alone. Both E1 antigen and defensin were present in the head and thoracic tissues of MRE/3'2J/ppA or MRE/3'2J/ppC-infected mosquitoes, but neither was detected in the reproductive tissues or Malpighian tubules (data not shown). Dissemination of virus in male mosquitoes infected as larvae was also examined, and E1 antigen and defensin expression were detected both in midgut and head tissues (data not shown).
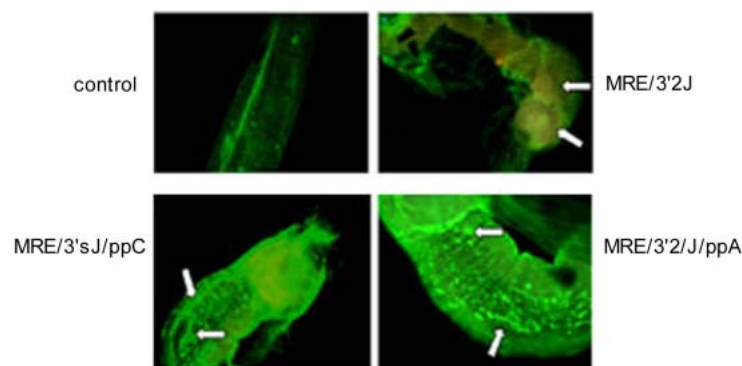
Defensin Expression in Mosquitoes Exposed to Recombinant Sindbis Viruses as Adults vs. Larvae

Ae. aegypti mosquitoes were exposed to viruses either in an infectious blood meal as adults, or in tissue culture flasks containing
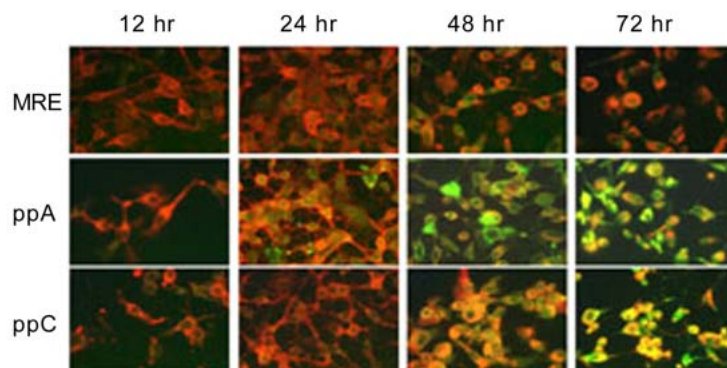
Figure 2. Expression of defensin in Sindbis recombinant virus-infected C6/36 Cells. Sindbis viruses MRE/3'2J (MRE), MRE/3'2/ppA (ppA), and MRE/3'2J/ppC (ppC) were inoculated into C6/36 cells (MOI = 3) and examined by indirect fluorescent assay for the expression of Sindbis E1 glycoprotein (Texas Red) and defensin peptide (FITC, green) at various hours after inoculation.

Figure 3. Defensin expression in midguts of MRE/3'2J/ppA or ppC virus-infected *Ae. aegypti* mosquitoes (FITC; patches of defensin peptide are indicated by arrows). Parental MRE/3'2J virus infected mosquitoes showed staining of only the E1 glycoprotein (Texas Red; arrow). Mosquitoes were exposed to virus-infected C6/36 cells as larvae, control group mosquitoes were exposed to uninfected C6/36 cells. Midguts of adult mosquitoes were removed by dissection 3 days after eclosion.

infected C6/36 cells as larvae. The MRE/3'2J parental SIN virus did not induce defensin expression in mosquito midgut tissues or hemolymph . Furthermore, northern analysis confirmed that oral infection of mosquitoes with MRE/3'2J, MRE/3'2J /ppA, or MRE/3'2J /ppC does not induce transcription of other immune peptides such as cecropin (data not shown). *Ae. aegypti* adults infected with MRE/3'2J/ppA or ppC viruses expressed defensin in the hemolymph 9-10 days after exposure (data not shown) with peak expression at 14 days. A representative western blot of hemolymph collected from adult mosquitoes 14 days after exposure to MRE/3'2J/ppA is shown in Figure 4. Adult mosquitoes exposed to a blood meal with lower viral titers had lower infection levels (approx. 6.0 log $_{10}$ TCID$_{50}$ per ml; infection level 40%; n = 20) compared to those exposed to higher viral titers (7.2 and 8 log $_{10}$ TCID$_{50}$ per ml; infection level 50 and 75%, respectively; n = 20).
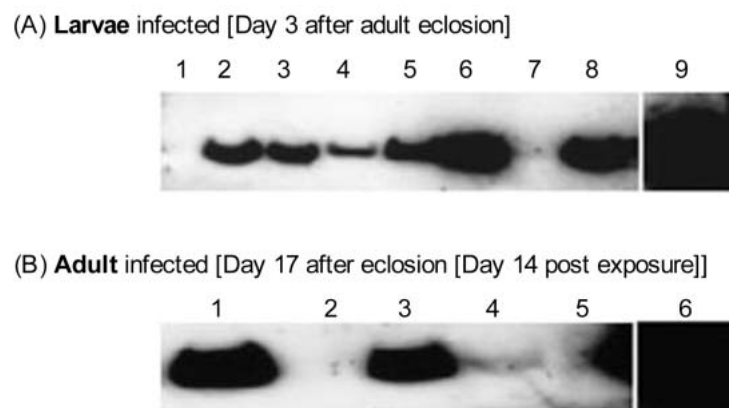
In contrast, mosquitoes that ingested recombinant SIN virus-infected C6/36 cells as larvae became infected and expressed defensin when they emerged as adults. Defensin expression was noticeable in the hemolymph by western blot 1 to 3 days after emergence (Figure 4) and reached the peak of expression 5 days post emergence (data not shown). Mosquitoes exposed as larvae showed higher levels of infectivity than those exposed at the adult stage. Approximately 80-88% of the mosquitoes exposed at the larval stage (n = 57) were infected as determined by tissue culture inoculation or via IFA. In comparison, the infection levels of mosquitoes exposed as adults (n = 48) ranged between 40 and 75%. From these data it is clear that exposing mosquitoes in the larval stage to dsSIN virus resulted in expression of the defensin gene at the time a female mosquito is most likely to take up an infectious blood meal in laboratory experimentation, 3-5 days after eclosion. Infection of larvae, therefore is the most biologically relevant procedure for studies of other genes of interest that potentially affect vector competence.

*Critical Conditions for Efficient Gene Expression in Mosquitoes*

The MOI used to infect C6/36 cells for exposure to larval mosquitoes was found to be critical for defensin expression. Fewer mosquitoes expressed defensin 3 days after emergence when C6/36

cells were infected at a MOI of 0.1 (10% for MRE/3'2J/ppA and 20% for MRE/3'2J/ppC; n = 10), as compared to those exposed to a MOI of 0.01 (70% for both MRE/3'2J/ppA and ppC; n = 10) (Figure 5A), even though the infection levels of mosquitoes remained comparable (80-85%, n = 20) as determined by virus isolation from mosquito carcasses in BHK cell cultures. This experiment was repeated three times and the level of defensin expression was consistently higher in the group infected with a MOI of 0.01. By 5 days after emergence, 75% of the adults infected with MRE/3'2J/ppA at a MOI of 0.01 (n = 8), and 63% of the mosquitoes infected with MRE/3'2J/ppC (n = 8) at the same MOI expressed defensin in the hemolymph (Figure 5B). In contrast, none of the mosquitoes expressed defensin in the hemolymph nor in the midguts when exposed to C6/36 cells infected with viruses at a MOI of 0.1 5 days after emerging (Figure 5B). Similarly, *in vitro* infection with MRE/3'2J/ppA at a MOI of 0.01 in C6/36 cells repeatedly resulted in a greater production of defensin than those inoculated with a MOI of 0.1 (Figure 6), even though the resulting viral titers were similar (7.8-8.2 log $_{10}$ TCID$_{50}$/ml 72 hours after infection).

The number of infected cells that larvae consume also influences subsequent defensin expression in all developmental stages (larvae, pupae, and adult) (Figure 7). In three experiments, maximum defensin expression was seen when 300 larvae were exposed to 2.5 to 3 x 10^7 MRE/3'2J/ppA-infected cells. In contrast, 200 larvae exposed to the same number of MRE/3'2J/ppC-infected cells provided the best defensin expression of defensin C, suggesting that there are functional differences between the two recombinant virus constructsLarvae were left in the flasks until all of the virus-infected cells were consumed; therefore, groups of 200 larvae should have consumed more infected cells than groups of 300. Table 1 summarizes the number of samples tested from three independent experiments. The numbers of defensin positive samples of larvae or pupae infected with MRE/3'2J/ppA or ppC viruses in groups of 200 or 300 larvae were significantly different (Mann-Whitney *U* test, *p* < 0.001). However, analysis of the hemolymph samples from adults between the two groups was not significant (for MRE/3'2J/ppA, *p* = 0.423; for MRE/3'2J/ ppC, *p* = 0.031). This density dependence for defensin expression was seen in each of the three repeated experiments, indicating that optimizing conditions are

(A) **Larvae** infected [Day 3 after adult eclosion]

(B) **Adult** infected [Day 17 after eclosion [Day 14 post exposure]]

Figure 4. Western blot analysis of defensin expression in hemolymph of adult *Ae. aegypti* infected as larvae (A) or adults (B) with MRE/3'2/ppA. Lane numbers represent individual mosquitoes. Lanes 10 (panel A) and 7 (panel B) contain 0.1 ng purified defensin as a positive control.
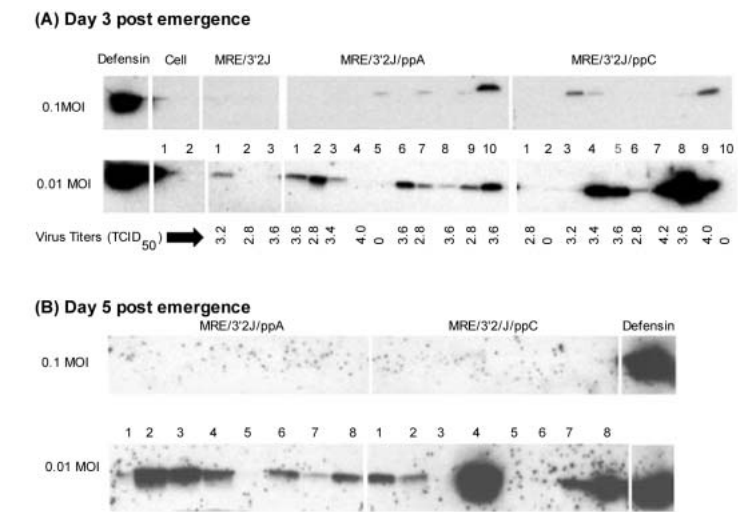
Figure 5. Expression of defensin in the hemolymph of adult *Ae. aegypti* exposed as larvae to parental MRE, MRE/3'2J/ppA and MRE/3'2J/ppC viruses-infected cells with different multiplicity of infection (MOI). Lane numbers represent different mosquitoes. . (A) Western analyses of hemolymph samples of adults collected 3 days after eclosion (cell control: uninfected C6/36 cells, MRE/3'2J, MRE/3'2J/ppA and MRE/3'2J/ppC groups). Individual mosquito carcasses from the lower MOI (0.01) group were titrated (TCID$_{50}$/ml) after the hemolymph was collected. (B) Hemolymph samples taken 5 days after eclosion from mosquitoes infected with MRE/3'2J/ppA and MRE/3'2J/ppC virus. For the positive control, 0.1 ng purified defensin was used.

crucial for expressing genes with the MRE/3'2J virus system.

Interfering Particles in the MRE/3'2J/ppA or ppC Viral Preparations

      Some infected mosquitoes (confirmed by virus isolation) did not express defensin in the hemolymph (Figure 5), nor in midgut tissues. In addition, in four repeated experiments (n = 32), when larvae were exposed to C6/36 cells infected with MRE/3'2J/ppA or MRE/3'2J/ppC with a high MOI (0.1), fewer adult mosquitoes (10-20%) expressed defensin as compared to those exposed to a lower MOI (0.01) (70-80%) 3 days after emergence. This suggests that

Table 1. The effect of mosquito numbers and potential virus uptake on defensin expression by MRE/3'2J/ppA or ppC virus in mosquitoes exposed as larvae to infected C6/36 cells.

|	MRE/3'2J/ppA			MRE/3'2J/ppC		
	Larvae[2]	Pupae[2]	Adults[2]	Larvae	Pupae	Adults
(a) 200 larvae[1]	6/22[3] (27%)	8/22 (36%)	13/20 (65%)	18/22 (82%)	21/22 (95%)	15/20 (75%)
(b) 300 larvae	21/22 (95%)	22/22 (100%)	16/20 (80%)	0/22 (0%)	1/22 (0.5%)	7/20 (35%)

[1] With each viral construct, group (a) represents 200 larvae fed on 2.7 x 10^7 virus-infected C6/36 cells, and group (b) represents 300 larvae.

[2] Larvae and pupae samples were collected 5 and 8 days respectively after exposure to virus-infected cells; adult hemolymph was collected 14 days after exposure (day 3 after eclosion).

[3] Values are numbers of defensin positive samples over total number of samples tested. Within treatment groups (different larvae numbers), comparisons of numbers of defensin positive samples were significantly different between groups (a) and (b) in larvae and pupae samples ($P< 0.001$), but were not significantly different in the adult samples ($P = 0.423$ for MRE/3'2J/ppA; $P = 0.031$ for MRE/3'2J/ppC) as determined by Mann-Whitney U test.
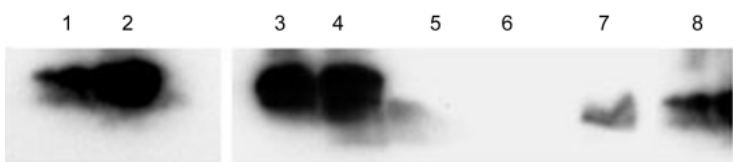
Figure 6. In vitro Defensin expression in C6/36 cells infected with MRE/3'2J/ppA at MOI of 0.01 (lanes 2 and 4) or MOI of 0.1 (lanes 1 and 3) 72 hours after inoculation. The supernatant of C6/36 cells infected with MRE/3'2J/ppA (lanes 1-4) or MRE/3'2J (lanes 5 and 6) was subjected to western analysis after removing the large molecules in the tissue culture medium using 30 KD molecular weight cutoff ultrafree-MC centrifugal filters (Millipore). 1 μg of protein was loaded in lanes 1-6. Lane 7 shows the hemolymph of an individual *Ae. aegypti* inoculated with bacteria. Lane 6 is 0.05 ng purified recombinant defensin.

MRE/3'2J parental viruses, mutant viruses, and/or other defective interfering particles influenced expression of the defensin gene. Hence, we compared our virus stock recovered from electroporated BHK cells to the viruses derived from re-inoculation of the original virus stock in BHK-21 cells at a MOI of 0.01. A higher percentage of MRE/3'2J parental or mutant virus existed in the original stock solution of MRE/3'2J/ppA than virus containing the ppA insert. Five of the 20 individual plaques (25%) isolated from the original stock of MRE/3'2J/ppA expressed the viral E1 protein but not defensin as determined by IFA, indicating that these plaques contained the parental virus or a deletion mutant. In comparison, 6 of 19 (31.5%) plaques generated from inoculation at a MOI of 0.1 contained parental viruses. In contrast, only 2 of the 20 plaques (10%) from viral stock generated with subsequent inoculation at MOI of 0.01 were of parental virus origin.

      The loss of the inserted gene could have occurred during *in vitro* transcription generating incomplete transcripts from the recombinant viral cDNAs. The resulting progeny viruses would be replication defective because the 3' non-coding region (NCR) would probably be missing; however, the presence of these defective interfering-like virus particles in the MRE/3'2J/ppA or MRE/3'2J/ppC viral stocks probably was minimal. Alternatively, the inserted gene could have been deleted during replication of the SIN viruses. TE/3'2J dsSIN viruses engineered to express CAT (Kamrud *et al.*, 1997) or GFP (Higgs *et al.*, 1996) that contained defective viral
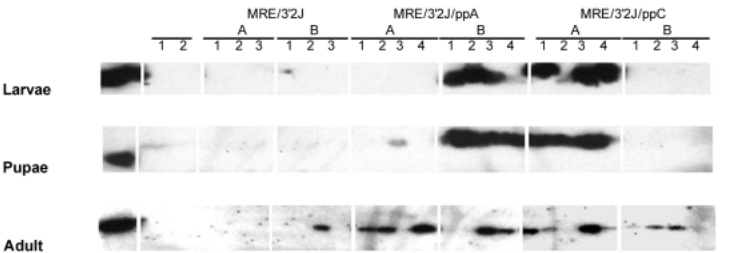
Figure 7. A comparison of mosquito numbers and potential virus uptake on defensin expression by western blot analysis. For each viral construct, group A represents 200 larvae and group B represents 300 larvae, fed on 2.7 x 10^7 virus infected-C6/36 cells. C6/36 cells were inoculated with viruses at a multiplicity of infection of 0.01 for both groups. For the positive control, 0.05 ng purified defensin was used. Larval (4th instar) and pupal samples were collected 5 and 8 days after exposure, respectively, and adult hemolymph was collected 3 days after emergence. The cell control group was exposed to uninfected C6/36 cells.

particles were subjected to sequence analyses. It was found that a section of the second subgenomic promoter and the inserted gene were missing, along with much of the 3' non-coding region and, therefore, seem to be mutant rather than parental viruses (unpublished data). In the case of MRE/3'2J/ppA or ppC, the high MOI used to re-amplify viruses in C6/36 cells prior to feeding mosquitoes might result in more mutant or parental MRE/3'2J virus in the inoculum due to the presence of viruses without defensin inserts in the virus stock. These parental or mutant viruses could compete with the MRE/3'2J/ppA or MRE/3'2J/ppC viruses for replication and increase the prevalence of viruses missing the defensin inserts in progeny viruses. In fact, western blot analysis of the supernatant from MRE/3'2J/ppA-infected C6/36 cells (Figure 6) showed that higher virus MOI (0.1) produced less defensin than lower virus MOI (0.01) 72 hours after inoculation, suggesting that higher numbers of mutant or parental viruses in the inoculum of high MOI had competed with MRE/3'2J/ppA resulting in a smaller amount of defensin expression.

Several strategies could be used to minimize the prevalence of parental MRE/3'2J or mutant viruses in virus stocks prepared from cDNAs. One possible approach is to re-inoculate the virus preparation in C6/36 cells at a low MOI (0.001-0.01). Another would be to modify the dsSIN vectors, so that the inserted heterologous gene is located toward the 5' end of the viral genome. In this case, the inserted gene would be under the control of the first subgenomic promoter, and the second subgenomic promoter would regulate the structural genes of the virus. This construct would minimize deletion mutations in the inserted gene and thus limit the possibility of interference from parental viruses or mutants.

The orally infectious dsSIN MRE/3'2J virus provides an excellent tool for expressing an inserted gene both *in vivo* and *in vitro*. Our data showed that endogenous defensin genes can be expressed in all developmental stages of *Ae. aegypti* (LVP) using the dsSIN MRE/3'2J vector to infect mosquitoes in the larval stage. Proper levels of virus used in the preparation of C6/36 cells, and the numbers of cells consumed by larvae are significant factors to consider when optimizing conditions for infecting mosquitoes to produce the desired levels of gene expression. Preliminary data also showed defensin expression in hemolymph in a different strain of *Ae. aegypti* (RexD) and another mosquito species, *Culex pipiens,* when MRE/3'2J/ppA- or MRE/3'2J/ppC-infected cells were fed to larvae (data not shown). Using this system, expression of genes of interest that may affect vector competence can be expressed at the time a mosquito would likely be fed on an infected host.

Acknowledgements

References

Adelman ZN, Blair CD, Carlson JO, Beaty BJ, Olson KE. 2001. Sindbis virus-induced silencing of dengue viruses in mosquitoes. . *Insect Biochemistry and Molecular Biology* 10:265-273.

Beerntsen BT, Christensen BM. 1990. *Dirofilaria immitis*: effect on hemolymph polypeptide synthesis in *Aedes aegypti* during melanotic encapsulation reactions against microfilariae. *Experimental Parasitology.* 71:406-414.

Chandler LJ, Beaty BJ, Baldridge GD, Bishop DH, Hewlett MJ. 1990. Heterologous reassortment of bunyaviruses in *Aedes triseriatus* mosquitoes and transovarial and oral transmission of newly evolved genotypes. *Journal of General Virology* 71:1045-1050.

De Lara Capurro M, Coleman J, Beerntsen BT, Myles KM, Olson KE, Rocha E, Krettli AU, James AA. 2000. Virus-expressed, recombinant single-chain antibody blocks sporozoite infection of salivary glands in *Plasmodium gallinacerum*-infected *Aedes aegypti. American Journal of Tropical Medicine and Hygiene* 62:427-33.

Gaines PJ, Olson KE, Higgs S, Powers AM, BJ Beaty, Blair CD. 1996. Pathogen-derived resistance to Dengue type 2 virus in mosquito cells by expression of the premembrane coding region of the viral genome. *Journal of Virology* 70:2132-2137.

Hahn CS, Hahn YS, Braciale TJ, Rice CM. 1992. Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. *Proceedings of the National Academy of Sciences USA* 89:2679-2683.

Harlow E., Lane D. 1988. Cell staining. In: *Antibodies. A laboratory manual*, 418. Cold Spring Harbor Laboratory: Cold Spring Harbor Press.

Higgs S, Traul D, Davis BS, Kamrud KI, Wilcox CL, Beaty BJ. 1996. Green fluorescent protein expressed in living mosquitoes – without the requirement of transformation. *Biotechniques* 21:660-664.

Higgs S, Oray CT, Myles KM, Olson KE, Beaty BJ. 1999. Infecting larval arthropods with a chimeric, double subgenomic Sindbis virus vector to express genes of interest. *Biotechniques* 27:908-911.

Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. 1999. Phylogenetic perspectives in innate immunity. *Science* 284:1313-1318.

Jiang W, Venugopal K, Gould EA. 1995 Intracellular interference of tick-born Flavivirus infection by using a single-chain antibody fragment delivered by recombinant Sindbis virus. *Journal of Virology* 69:1044-1049.

Johnson BW, Olson KE, Allen-Miurs T, Rayms-Keller A, Carlson JO, CJ Coates, Jasinskiene N, James AA, Beaty BJ, Higgs S. 1999. Inhibition of luciferase expression in transgenic *Aedes aegypti* mosquitoes by Sindbis virus expression of antisense luciferase RNA. *Proceedings of the National Academy of Sciences USA* 96:13399-13403.

Kamrud KI, Olson KE, Higgs S, Powers AM, Carlson JO, Beaty BJ. 1997. Detection of expressed chloramphenicol acetyltransferase in the saliva of *Culex pipiens* mosquitoes. *Insect Biochemistry and Molecular Biology* 27:423-429.

Lowenberger CA, Bulet P, Charlet M, Hetru C, Hodgeman B, Christensen BM, Hoffmann JA. 1995. Insect immunity: isolation of three novel inducible antibacterial defensins

from the vector mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology* 25:867-873.

Lowenberger CA, Ferdig MT, Bulet P, Khalili S, Hoffmann JA, Christensen BM. 1996. *Aedes aegypti*: induced antibacterial proteins reduce the establishment and development of *Brugia malayi*. *Experimental Parasitology* 83:191-201.

Lowenberger CA, Kamal S, Chiles J, Paskewitz S, Bulet P, Hoffmann JA, Christensen BM. 1999a. Mosquito-*Plasmodium* interactions in response to immune activation of the vector. *Experimental Parasitology* 91: 59-69.

Lowenberger CA, Smartt CT, Bulet P, Ferdig MT, Severson DW, Hoffmann JA, Christensen BM. 1999b. Insect immunity: molecular cloning, expression and characterization of cDNAs and genomic DNA encoding three isoforms of insect defensin in *Aedes aegypti*. *Insect Molecular Biology* 8:107-118.

Olson KE, Myles KM, Seabaugh RC, HiggsS, Carlson JO, Beaty BJ. 2000. Development of a Sindbis virus expression system that efficiently expresses green fluorescent protein in midguts of *Aedes aegyti* following *per os* infection. *Insect Molecular Biology* 9:57-65.

Powers AM, Kamrud KI, Olson KE, Higgs S, Carlson JO, Beaty BJ. 1996. Molecularly engineered resistance to California serogroup virus replication in mosquito cells and mosquitoes. *Proceedings of the National Academy of Sciences USA* 93:4187-4191.

Rutledge LC, Ward AR, Gould, DJ. 1964. Studies on the feeding response of mosquitoes to nutritive solutions in a new membrane feeder. *Mosquito News* 24: 407-419.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning. A laboratory manual*, A.2. Cold Spring Harbor Laboratory: Cold Spring Harbor Press.

Seabaugh RC, Olson KE, Higgs S, Carlson JO, Beaty BJ. 1998. Development of a chimeric Sindbis virus with enhanced *per Os* infection of *Aedes aegypti*. *Virology* 243:99-112.

Shiao SH., Higgs S, Adelman Z, Christensen BM, Liu AH, Chen CC. 2001. Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito *Armigeres subalbatus*. *Insect Molecular Biology* (In press).

Taylor RM, Hurlbut HS, Work TH, Klingston JR, Frothingham TE. 1955. Sindbis virus: a newly recognized arthropod-transmitted virus. *American Journal of Tropical Medicine and Hygiene* 4:844-862.