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

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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. albopictus* 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  
TCID₅₀ 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 circumzoozoite 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 (Hoffman 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* (DH7 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 x 10⁷ 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 cm² tissue culture flasks. Viruses were harvested from BHK cells and titrated (plaque forming unit and tissue culture infectious dose 50% end-points (TCID₅₀) ) 24 hours after transfection. Approximately 5-8 x 10⁷ plaque forming units or 7.2-8 log10 TCID₅₀ 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., 1999) 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 28°C 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 28°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 28°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. Mid guts 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
mosquitoes were ground in microcentrifuge tubes in 100 µl 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 fluorescein-isothiocyanate (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 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.

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.
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<sub>10</sub> TCID<sub>50</sub> per ml; infection level 40%; n = 20) compared to those exposed to higher viral titers (7.2 and 8 log<sub>10</sub> TCID<sub>50</sub> per ml; infection level 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<sub>10</sub> TCID<sub>50</sub>/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<sup>7</sup> 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 constructs. Larvae 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
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 virus-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 (TCID₅₀/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.

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

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

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

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.

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<th>MRE/3'2J/ppA</th>
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<tr>
<td></td>
<td>Larvae</td>
<td>Pupae</td>
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<tr>
<td>(a) 200 larvae</td>
<td>6/22 (27%)</td>
<td>8/22 (36%)</td>
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<tr>
<td>(b) 300 larvae</td>
<td>21/22 (95%)</td>
<td>22/22 (100%)</td>
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1 With each viral construct, group (a) represents 200 larvae fed on 2.7 x 10⁷ 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.
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.

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