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Antiviral Research 76 (2007) 222–231
Antiviral Research
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Construction and characterization of a stable subgenomic dengue virus type 2 replicon system for antiviral compound and siRNA testing

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Received 28 November 2006; accepted 8 June 2007

Abstract

Self-replicating, non-infectious flavivirus subgenomic replicons have been broadly used in the studies of trans-complementation, adaptive mutation, viral assembly and packaging in Kunjin, yellow fever and West Nile viruses. We describe here the construction of subgenomic EGFP- or *Renilla* luciferase-reporter based dengue replicons of the type 2 New Guinea C (NGC) strain and the establishment of stable BHK21 cell lines harboring the replicons. In replicon cells, viral proteins and RNAs are stably expressed at levels similar to cells transfected with the full length NGC infectious RNA. Furthermore, the replicon can be packaged by separately transfected C (core)-prM (pre-membrane)-E (envelope) polyprotein construct. The replicon cells were subjected to treatment with several antiviral compounds and inhibition of the replicon was observed in treatment with known nucleoside analog inhibitors of NS5 such as 2′-*C*-methyladenosine ($EC_{50} = 2.42 \pm 0.59$ μM), or ribavirin ($EC_{50} = 6.77 \pm 1.33$ μM), mycophenolic acid ($EC_{50} = 1.31 \pm 0.27$ μM) and siRNA against NS3. The BHK-replicon cells have been stably maintained for about 10 passages without significant loss in reporter intensity and are sufficiently robust for both research and drug discovery.

Keywords: Flavivirus; Dengue; Replicon; NS5 inhibitor; High-throughput screening

1. Introduction

Members of the genus *Flavivirus* (family *Flaviviridae*) are positive-stranded RNA viruses. This family of virus include important human pathogens such as dengue virus, yellow fever virus, Japanese encephalitis virus, West Nile virus and tick-borne encephalitis virus. The four genetically related but serologically distinct serotypes of dengue virus cause dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Subgenomic replicon constructs of the *Flaviviridae* contain all of the genetic elements needed for amplification in susceptible hosts but lack the major part of the genes encoding the structural proteins. The RNAs consequently replicate but are not packaged into viral particles. Because of their non-infectious nature, replicons are important tools in the study of viral replication. In *Flaviviridae*, Kunjin virus replicon has been well established to characterize adaptive mutations (Liu et al., 2004), trans-complementation (Khromykh et al., 2000), virus packaging and assembly (Harvey et al., 2004). West Nile virus replicon was constructed and developed into a high throughput assay for screening inhibitors of viral replication (Lo et al., 2003b; Rossi et al., 2005; Shi et al., 2002). Similarly, yellow fever virus replicon was also made and used in trans-complementation and packaging studies (Jones et al., 2005a). In dengue, the type 2 New Guinea C (NGC) strain virus was constructed into a subgenomic replicon (Pang et al., 2001a,b). The replicon could be detected via immunofluorescence after 8 days in less than 1% of the total population of the monkey LLC-MK2 cells. Recently, another strain of dengue 2, the 16681 strain was used to construct a luciferase-reporter containing replicon and

Abbreviations: DEN, dengue; DEN-2, dengue virus type 2; NGC, New Guinea C; NS, non-structural protein; C, core; E, envelope; EGFP, enhanced green fluoresecent protein; prM, pre-membrane; FCS, fetal calf serum; HRP, horse radish peroxidase; PBS, phosphate-buffered saline; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration

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doi:10.1016/j.antiviral.2007.06.007

was expressed in BHK21 and C6/36 cells in a transient manner (Alvarez et al., 2005; Holden et al., 2006). Replicon activity persisted for up to 120 h post-transfection. We describe here the construction of a self-replicating dengue 2 replicon of the NGC strain, containing an EGFP or *Renilla* luciferase-reporter and a puromycin resistance gene. Using puromycin selection, we established BHK21 cell lines stably expressing the replicons. Replicon activities in BHK21 cells are stable up for about 10 passages thus enabling classical studies of dengue viral replication. Additionally, the replicon can also be used for screening antiviral compounds and for profiling escape mutants arising from resistance to antiviral lead compounds under development in drug discovery.

2. Materials and methods

2.1. Cell culture, antibody and reagents

BHK21 cells (BHK21 c13) were purchased from ATCC and maintained in DMEM containing 10% FCS. Rabbit polyclonal antisera against the DEN-2 NS2B-NS3 protein, produced by injecting the fusion protein linking NS2B and NS3 via a flexible linker (G_4SG_4) was made in-house. Antibody against the DEN-2 NS4B protein was made in-house and described in Umareddy et al. (2006). Mouse polyclonal antiserum against the DEN-2 NS5 protein (Johansson et al., 2001) was a kind gift from James Cook University. γ-Tubulin antibody was purchased from

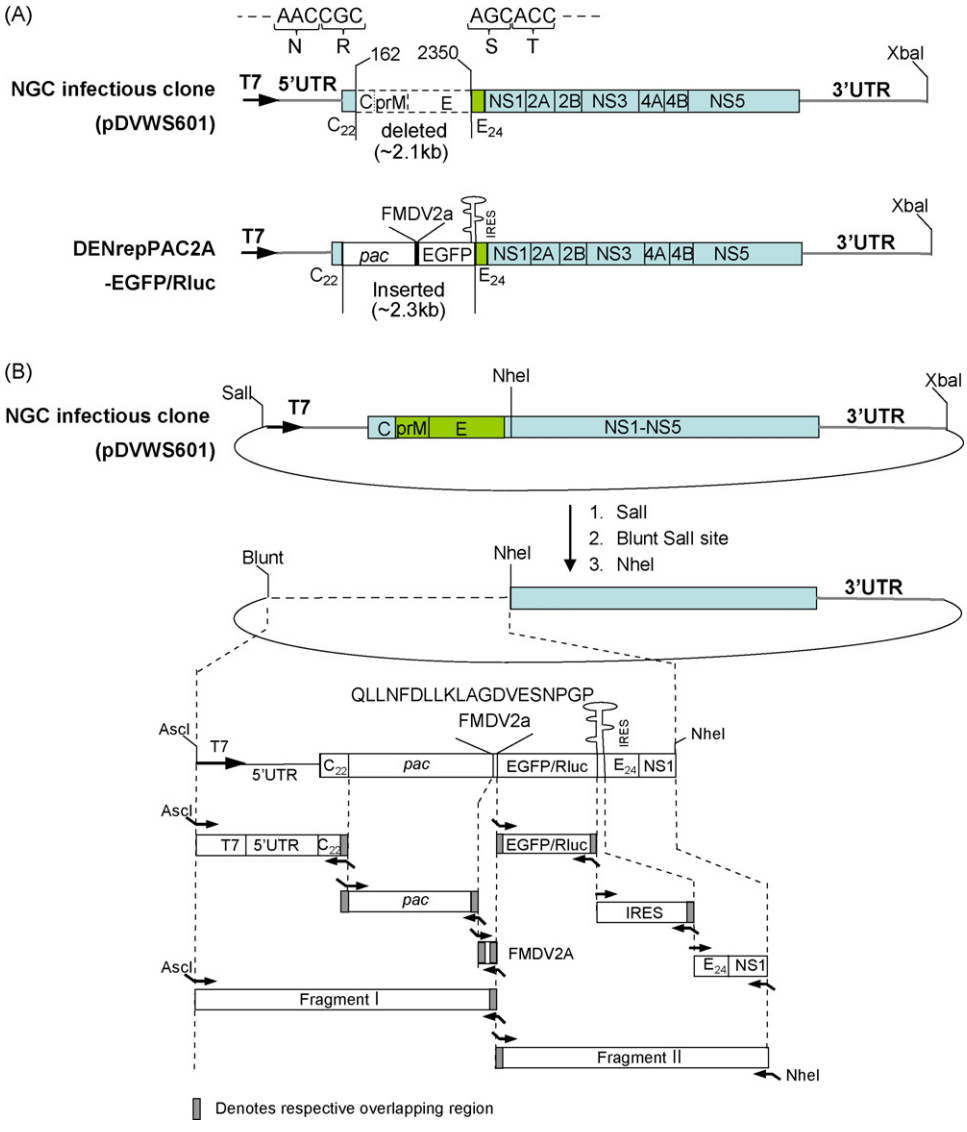


Fig. 1. (A) The schematic representation of the EGFP- and Rluc-replicon. From the full length DEN-2 NGC infectious cDNA clone pDVWS601, a 2.1 kb fragment spanning nucleotides 162–2350, encompassing the large part of C, E and the entire prM gene, was deleted, leaving sequence encoding the N-terminal 22 amino acids of the C protein (C_{22}), ending with Asparagine (N) and Arginine (R), and the C-terminal 24 amino acids of E (E_{24}), starting with serine (S) and threonine (T). The deleted sequence was replaced by a 2.3 kb fragment containing a gene fusion encoding puromycin resistance (puromycin-*N*-acetyl-transferase, or *pac*), a FMDV2A cleavage site, EGFP/Rluc and an ECMV (Encephalomyocarditis virus) IRES (internal ribosomal entry site). (B) The construction and ligation of the cassette containing the *pac* gene, a FMDV2A cleavage site, the EGFP/Rluc gene and an IRES. The cassette was produced by overlapping PCR as described in Section 2. The resulting cassette was then digested with NheI. NGC infectious clone vector pDVWS601 was digested with SalI, made blunt with Klenow and digested subsequently with NheI. The cassette and the vector were then ligated to form the EGFP/Rluc-replicon.

Sigma. Anti-mouse and anti-rabbit HRP antibodies were purchased from Santa Cruz. 2′-*C*-methyladenosine was synthesized in-house. All other reagents were obtained from Fisher Scientific and chemicals from Sigma, unless otherwise mentioned.

2.2. EGFP- and Renilla luciferase-replicon construction

The backbone of the EGFP-replicon was pDVWS601 plasmid which contains an infectious cDNA clone corresponding to the genome of the DEN-2 strain NGC in the vector pWSK29 (Gualano et al., 1998). To construct the replicon, a 2.1 kb fragment was removed from pDVWS601 and replaced with a new cassette (2.3 kb) containing genes encoding puromycin resistance (*pac*) and EGFP/*Renilla* luciferase (Rluc) and an IRES element (Fig. 1A). The new cassette was constructed via PCR assembly of several different PCR fragments (Fig. 1B; see Table 1 for complete list of primers). Initially, primer pair AscI-T7-F (forward) and C22-R (reverse) were used to amplify the region encompassing the T7 promoter, 5′UTR and sequence encoding the N-terminal 22 amino acids of the C protein. The *pac* gene was then amplified from the plasmid pIRESPuro3 (BD Biosciences) and the FMDV2A cleavage site amplified by overlapping PCR using the FM2A-F and FM2A-R primers. The resulting three fragments (T7-C22, *pac* and FMDV2A) were then joined by overlapping PCR to form fragment I (Fig. 1B). The EGFP gene was amplified from the plasmid pEGFP-1 (BD Biosciences), Rluc gene was amplified from the plasmid phRL-CMV (Promega) and an IRES element amplified from the plasmid pIRESPuro3 (BD Biosciences). A STOP codon was incorporated at the end of both EGFP and Rluc gene. The primer pair E24-F and NS1-Nhe I-R was used to amplify a fragment containing the last 72 nucleotides of the E gene and the first 128 nucleotides of the NS1 gene from pDVWS601. An ATG start codon was inserted before E24. The resulting three fragments (EGFP/Rluc, IRES and E24-NS1-NheI) were joined by overlapping PCR to form fragment II (Fig. 1B). Fragments I and II were then joined by overlapping PCR to generate the final 2.3 kb cassette which was digested with NheI. pDVWS601 was also digested with SalI and blunt-ended with Klenow digestion (New England Biolabs) followed by digestion with NheI. Both the PCR fragment and the vector were gel purified, ligated and positive clones isolated and sequenced verified.

2.3. RNA transcription, electroporation and selection of puromycin-resistant EGFP-replicon containing cells

Escherichia coli Top10 cells (Invitrogen) transformed with either the DENrepPAC2A-EGFP, DENrepPAC2A-Rluc or pDVWS601 were grown in LB medium containing 100 μg/ml carbenicillin (Invitrogen) at 30 °C overnight. Plasmid DNA was purified by Maxiprep (Qiagen) and linearised by XbaI digestion. DNA was phenol-chloroform extracted twice, precipitated and used as a template for *in vitro* transcription, in the presence of m^7GpppA (Promega), using the T7 Ribomax system (Promega) according to the manufacturer's protocol. After the transcription reaction, the template DNA was removed by a 30 min

Table 1
Oligonucleotide primers employed for PCR

Region	Designation	Sequence 5′–3′	Sequence designation, restriction enzyme site and introduced sequences
T7 to C22	AscI-T7-F	A<u>GGCGCGCC</u>**TAATACGACTCACTATA**G	AscI site (underlined), T7 (bold)
	C22-R	**GGGCTTGTACTCGGTCAT**<u>GCGGTTTCTCTCGCGTTTC</u>	5′ nt 1–18 of *Pac* (bold), C22 nt 162–144 (underlined)
Pac	*pac*-F	**GAAACGCGAGAGAAACCGC**<u>ATGACCGAGTACAAGCCCAC</u>	C22 nt 144–162 (bold), *Pac* 5′ nt 1-20 (underlined)
	pac-R	**GTCGAAGTTCAGAAGCTG**<u>GGCACCGGGCTTGCGGGTC</u>	FM2A 5′ nt 1–18 (bold), *Pac* 3′ 19 nt (underlined)
FMDV2A	FM2A-F	CAGCTTCTGAACTTCGAC**CTCCTCAAGTTGGCGGGAGACGTTGAG**	FM2A overlapping (bold)
	FM2A-R	AGGCCCAGGGTTGGA**CTCAACGTCTCCCGCCAACTTGAGGAG**	FM2A overlapping (bold)
EGFP	EGFP-F	**GAGTCCAACCCTGGGCCT**<u>ATGGTGAGCAAGGGCGAG</u>	FM2A (bold), EGFP 5′nt 1–18 (underlined)
	EGFP-R	**GGGGGAGGGAGAGGGGCG***TCA*<u>CTTGTACAGCTCGTCCATGC</u>	IRES (bold), EGFP 3′ 20 nt (underlined) including STOP codon (Italic and underlined)
IRES	IRES-F	CGCCCCTCTCCCTCCCCC	IRES 5′ nt 1–18
	IRES-R	**GTGACACAGACAGTGAGGTGCT*CAT***<u>GGAAGGTCGTCTCCTTGTG</u>	E 3′ nt 2371–2350 (bold), ATG (***CAT***), IRES 3′ nt 19 (underlined)
E24 to NS1 NheI	E24-F	AGCACCTCACTGTCTGTGTCAC	E 3′ 2371–2350
	NS1-NheI-R	GCTGA<u>AGCTAGCT</u>TTGAAGGGGAT	NS1 2555–2543, NheI (underlined)
RLUC	RLUC-F	**GAGTCCAACCCTGGGCCT**<u>ATGGCTTCCAAGGTGTAC</u>	FM2A (bold), Rluc 5′ nt 1–18 (underlined)
	RLUC-R	**GGGAGGGAGAGGGGCG*TTA***<u>CTGCTCGTTCTTCAGCAC</u>	IRES 5′ 16 nt, STOP (***TTA***), Rluc 3′ 21 nt (underlined)

F: Forward; R: reverse.

DNase I digestion and the resulting RNA was extracted twice with phenol-chloroform, precipitated with sodium acetate and ethanol, washed with 70% ethanol, resuspended in RNase-free water and quantitated by spectrophotometry.

For RNA electroporations, 2×10^6 BHK21-C13 cells were washed twice before being resuspended in 0.4 ml of room-temperature PBS containing 10 μg of RNA. The cells were pulsed three-times at 0.85 kV/25 μF in 0.4-cm cuvettes using the GenePulser apparatus (Bio-Rad). Electroporated cells were plated into two wells of a six-well plate and grown in complete growth media (10% FCS, RPMI 1640). Twenty-four hours post-electroporation, fresh media containing 5 μg/ml of puromycin was added.

To select for puromycin-resistant cells, media were replaced every 3–4 days. After 7 days, individual foci of puromycin-resistant fluorescent cells were picked and expanded in the presence of puromycin and frozen in 10% dimethyl sulphoxide in liquid nitrogen.

2.4. EGFP-replicon packaging by C-prM-E

The DEN-2 TSV01C, prM and E genes, encoding a polyprotein, were cloned into the mammalian expression vector pXJ by Sandra Bamberg (NITD). A fragment containing the C-prM-E gene sequence was PCR amplified from pDVWS601 using the sense primer: 5′-ATAAGAATGCGGCCGCAGTTGTTA-GTCTACGTGGACCGAC-3′ (sequence corresponding to DEN-2 nucleotides 1–24 is underlined), and antisense primer 5′-CCGCTCGAGTTAGGCCTGCACCATAACTCCCAAATAC-3′ (sequence corresponding to the last 25 nucleotides of the DEN-2 E gene is underlined). The PCR fragment was cloned into the pXJ vector (Zheng et al., 1992) using NotI and XhoI sites. The expression of the protein was verified by Western blot (data not shown). Clone #2.6 of the stable DENrepPAC2A-EGFP containing BHK21 cells were plated into six-well plates and transfected with 1 μg of pXJ-C-prM-E plasmid or the empty vector using lipofectamine 2000 (Invitrogen). Sixteen hours post-transfection, the media was changed to RPMI (2% FCS) without puromycin. The culture supernatant of the transfected cells was harvested 48 h post-transfection. Cell debris was removed by centrifugation and 1 ml of the supernatant was transferred onto native BHK21 cells. Cells were incubated without shaking for 4 h after which time the media was replaced with fresh complete media and re-incubated for 3 days and observed by fluorescence microscopy.

2.5. Detection of proteins expressed by the replicon using Western blot, immunofluorescence and electron microscopy

The replicon containing cells or BHK21 cells transfected with the DEN-2 NGC infectious clone (pDVWS601) were harvested in m-RIPA (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate) buffer containing complete protease inhibitor cocktail (Roche). The protein concentration was determined using the Bradford method (Biorad) (Bradford, 1976) and 25 μg of each sample was analyzed by SDS-PAGE, transferred to nitrocellulose membranes and detected with anti- NS2B/NS3, NS4B, NS5 and γ-tubulin antibodies. For immunofluorescence, the BHK21 clone #2.6 containing DENrepPAC2A-EGFP were fixed in 4% paraformaldehyde and permeablized with 0.1% Triton X-100. Cells were then incubated with anti-NS2B/NS3 rabbit polyclonal antibodies and detected using tetramethylrhodamine isothiocyanate (TRITC) labeled anti-rabbit antibodies (Jackson Immunolab). For electron microscopy, cells were fixed in 2.5% glutaraldehyde, scraped, pelleted, Epon embedded and sectioned for analysis using a JEOL 1010 transmission electron microscope at the National University of Singapore Microscope Facility.

2.6. Real-time PCR

RNA was extracted from one million replicon containing cells or cells transfected with infectious clone NGC RNA using the RNeasy kit (Qiagen) and quantified by spectrophotometry. cDNA synthesis was performed using a Superscript III kit (Invitrogen) with 300 ng (∼2% of total) of extracted RNA and the specific antisense primer binding to the NS1 gene (nts 2958–2980; 5′-CCGCTGACATGAGTTTTGAGTC-3′). Subsequently, real-time PCR reaction was performed using the Fast Start Master^{PLUS} SYBR Green I kit (Roche Diagnostics) in a Roche Light Cycler 2. The reaction was carried out as follows: 1 μl (out of 20 μl or 5%) of the cDNA was amplified with the following primer pair: sense primer (nts. 2685–2707, 5′-CCGCTGACATGAGTTTTGAGTC-3′) and antisense primer (nts. 2958–2980, 5′-YATGACAGGAGACATCAAAGGA-3′, Y being either a C or a T) using the following cycling regime: 95 °C for 10 min (1 cycle), 95 °C for 5 s, 55 °C for 5 s and 72 °C for 20 s (40 cycles). Results were analyzed by Light Cycler software version 4.0. A standard curve was routinely plotted in every experiment using 0.01, 0.1, 1 and 10 ng of DNA of NGC infectious clone plasmid (pDVWS601). With the known size of the plasmid (16.4 kb), the theoretical copy number was calculated and a standard curve was established between the Cp value and copy number. Assuming optimal efficiency of reverse transcription, the Cp values obtained for different clones of replicon cells in a PCR reaction could be extrapolated to copy number using the standard curve. From the copy number in each PCR reaction, the copy number per cell was then calculated with the following formula: copy number per PCR reaction $\times 20 \times 50/10^6$.

2.7. Dose–response of NS5 inhibitor and antiviral compounds in dengue replicon and siRNA inhibition

Selected clones of EGFP and Rluc reporter containing replicon cells were plated at a density of 1×10^4 cells per well in a 96-well plate. The next day, normal culture media (RPMI with 10% FCS) was changed to RPMI with 2% FCS and compound added in 2× serial dilutions in the range of 0.195–100 μM. For EGFP-replicon cells, after 72 h incubation, the culture media was aspirated and 100 μl of PBS containing 0.5% Triton X-100 was added to each well, incubated at 37 °C for 1 h and the fluorescent intensity was measured using a Tecan Safire^2 plate reader with excitation set at 488 nm (±20 nm) and emission set at 530 nm (±20 nm). For Rluc-replicon cells, EnduRen^{TM}

(Promega) was added at 1:1000 dilution and incubated for 1 h before the luminescence was read. Using Prism v.4 (Graphpad software, San Diego, CA) software, the relative fluorescent unit (RFU) or luminescence reading was plotted against the log transformation of the concentration of the compound and a sigmoidal curve fit with variable slope was done to obtain the effective concentration (EC_{50}) value. For the same range of compound concentration, cell viability was also measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega). This assay determines cell viability via the reduction of an MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) tetrazolium compound to a formazan product, which could be detected at 490 nm. Ten microliters of the CellTiter solution was added to 50 μl of media after compound incubation incubated for 1 h at 37 °C, and the absorbance measured at 490 nm. Alternatively, cell viability was measured by CellTiter-Glo luminescence cell viability assay (Promega) for ATP detection in live cells. siRNA experiment was performed in EGFP containing replicon cells. siRNA against EGFP was from Dharmacon and NS3 siRNA was synthesized by Dharmacon (sense: GAUGGAGCCUAUAGAAUCAUU, antisense: UGAUUCUAUAGGCUCCAUCUU). siRNA was delivered using lipofectamine 2000 (Invitrogen). Different passage of cells were used up to passage 13 before the reporter signal decreases.

3. Results

3.1. Construction of dengue 2 subgenomic replicon and development of BHK21 cell lines stably expressing replicons

Similar to other *Flaviviridae* members, dengue virus possesses a 11 kb positively strand 5′-capped RNA genome containing three structural genes and seven non-structural genes in the order of C (core)-prM (pre-membrane)-E (envelope)-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Lindenbach and Rice, 2003). After virus entry and fusion with host cells, the RNA genome is released and replicated on the ER membrane in the replication complex (Uchil and Satchidanandam, 2003).

In order to construct the replicon, a fragment encompassing almost the entire structural gene region was deleted from the DEN-2 NGC infectious clone pDVWS601 (Gualano et al., 1998) (Fig. 1A). Only the nucleotides encoding the N-terminal 22 amino acids of the C protein which contains the cyclization motif important for NS5 dependent RNA replication (Lo et al., 2003a; You and Padmanabhan, 1999), and the last 24 amino acids of the E protein, containing the transmembrane region and the NS1 signal sequence (Chambers and Rice, 1987) remained. The deleted structural genes were replaced by a new cassette containing the gene encoding puromycin resistance (puromycin-*N*-acetyltransferase, or *pac*), a FMDV2A (Foot-and-mouth-disease virus 2A) cleavage site, followed by an EGFP reporter gene or *Renilla luciferase* gene and an ECMV (Encephalomyocarditis virus) IRES (internal ribosomal entry site) (Fig. 1A). The FMDV2A cleavage site was inserted to ensure that the puromycin resistance gene product and EGFP were properly processed (Varnavski et al., 2000), while positioning the IRES upstream of the non-structural genes ensured adequate translation of dengue virus non-structural proteins (see Fig. 1B and Section 2 for detailed construction).

EGFP-replicon (DENrepPAC2A-EGFP) or Rluc-replicon (DENrepPAC2A-Rluc) DNA was then transcribed *in vitro* and the resulting RNA transcripts electroporated into BHK21 cells. In a transient transfection experiment, replicon expression was monitored daily by fluorescence microscopy. We observed strong EGFP signal on days 1 and 2 post-electroporation in about 10% of the cells. In order to select cells stably maintaining the replicon, puromycin was added to the growth media 48 h post-transfection for 2–3 weeks. Various clonally derived BHK21 cell lines were obtained and expanded for further characterization.

3.2. Characterization of the replicon containing BHK21 cell lines

We first checked the expression of non-structural proteins in EGFP- and Rluc-replicon containing cell lines. The presence of the NS3 protease, NS4B protein and NS5 polymerase were detected in three different clones of EGFP-replicon containing cells (Fig. 2A) and in eight different clones of Rluc-replicon containing cells (Fig. 2B). The expression levels of NS proteins varied in different clones and the high expressing clones such as #2.6, #18 of the EGFP-replicon cell and #29 of the Rluc-replicon cell showed equal or higher amount of NS3 and NS5 compared to BHK-21 cells transfected with same amount of RNA (5 μg) of NGC infectious clone at day 7 post-transfection.

Real-time PCR using a pair of NS1 gene specific primers was carried out to determine the copy number of the replicon in different clones relative to the copy number of the viral genome in infectious clone RNA-transfected cells. The replicon copy number was calculated according to a standard curve established using a known amount of full length NGC infectious clone DNA plasmid in the real-time PCR reaction (see Section 2). For clones #2.6, #18 and #29, the number of replicon copies per cell was estimated to be about 15,000, in the same range as the copy number of viral genome in BHK21 cells 7 days after transfection with 5 μg of RNA of the NGC infectious clone (Fig. 2C and D).

To further compare the replicon containing cells to virus infected cells, the ultrastructure of the EGFP-replicon containing cell clone #2.6 and virus-infected cells was examined by electron microscopy. As shown in Fig. 3, "vesicular packets" (white arrow heads) typical of flavivirus replication were found in NGC-infected BHK cells (Uchil and Satchidanandam, 2003). The same "vesicular packets" were also found in the EGFP-replicon containing cells, confirming the presence of viral replication.

Since the EGFP-replicon contained the dengue genome except the structural genes, we examined whether *trans* expression of the structural genes allowed assembly, packaging and production of viral particles. The EGFP-replicon cell line (clone #2.6) was transfected with either a mammalian expression vector containing the DEN-2 TSV01 C-prM-E genes (pXJ-C-prM-E; see Section 2) or the empty vector pXJ. Two days later, the

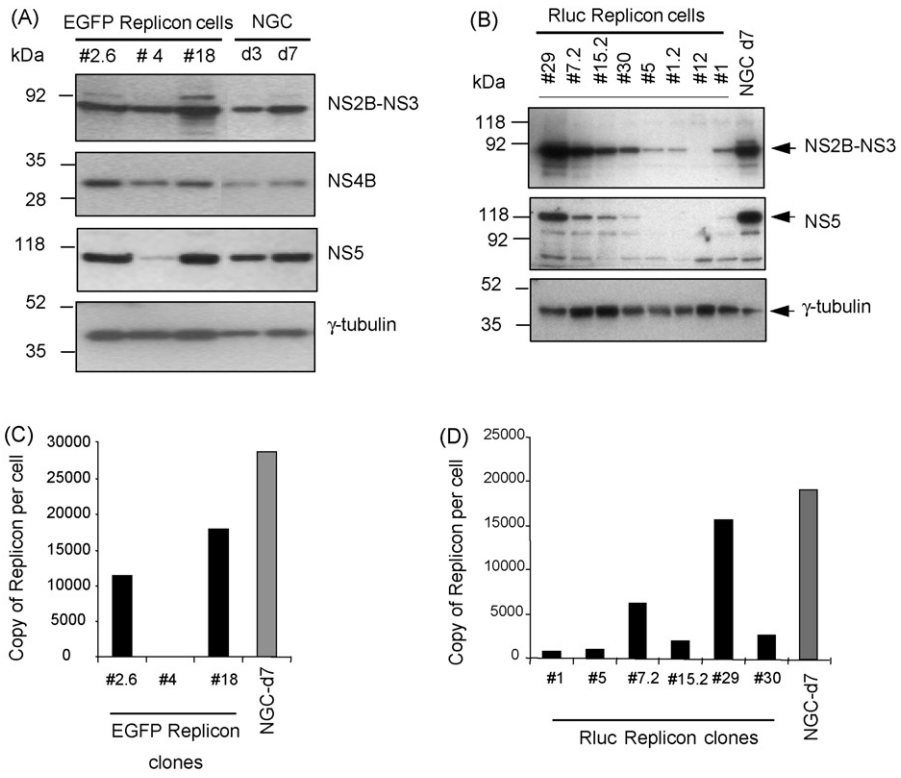


Fig. 2. (A) Three clonally derived BHK21 EGFP-replicon containing cell lines (#2.6, #4 and #18) were analyzed by immunoblot for the presence of NS2B-NS3, NS4B and NS5 proteins at passage 8. RNA transcribed *in vitro* from NGC infectious clone was transfected into BHK cells and cell lysates prepared 3 or 7 days post-transfection (d3, d7). The cell lysates were analyzed by immunoblot using the indicated panel of antibodies and γ-tubulin was used as an internal control. (B) Eight different clones of BHK21 Rluc-replicon containing cell lines (#29, #7.2, #15.2, #30, #5, #1.2, #12, #1) at passage 6 were analyzed by immunoblot for the presence of NS2B-NS3 and NS5. The day 7 post-NGC infectious clone RNA transfected BHK cell lysate (d7) (as in A) was used as a control. (C) Real-time PCR reaction was done on EGFP-replicon clones #2.6, #4 and #18 from passage 8 cell culture. As comparison, real-time PCR was done on BHK21 cells transfected with NGC infectious clone RNA for 7 days (NGC-d7) with the same amount of total extracted RNA in the reaction. The copy number per cell was calculated according to a standard curve and dilution factors (see Section 2). (D) Real-time PCR was performed Rluc-replicon clones #1, #5, #7.2, #15.2, #29 and #30 from passage 13 cell culture. The same control was used as in C (NGC-d7).

supernatants from the respective transfectants were collected and transferred onto naive BHK21 cells. After 3 days of growth, BHK21 cells incubated with supernatant from the pXJ-C-prM-E transfected replicon cells showed the presence of EGFP, while cells incubated with supernatant from the pXJ transfected replicon cells did not produce any EGFP signal. This result suggested that the EGFP replicon had been successfully packaged by the C-prM-E proteins produced *in-trans* and could carry out infection

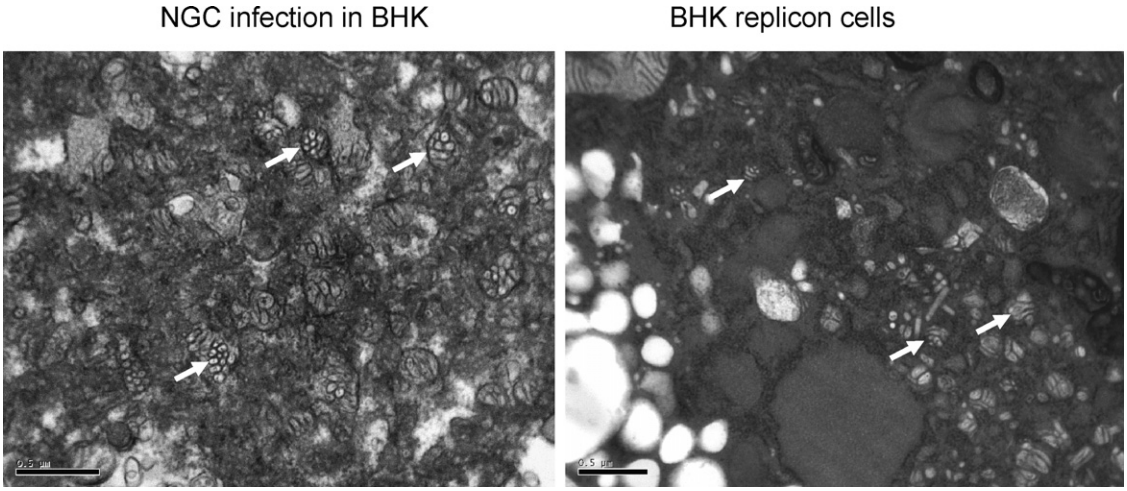


Fig. 3. EGFP-replicon clone #2.6 from passage 8 culture was examined by electron microscopy. As a control, BHK cells were also infected with NGC virus at a M.O.I of 10 and sampled 2 days post-infection. Both infected BHK21 and EGFP-replicon containing cells were fixed and processed for electron microscopy. On both pictures, the typical "vesicular packets" induced by viral replication are indicated by white arrows.

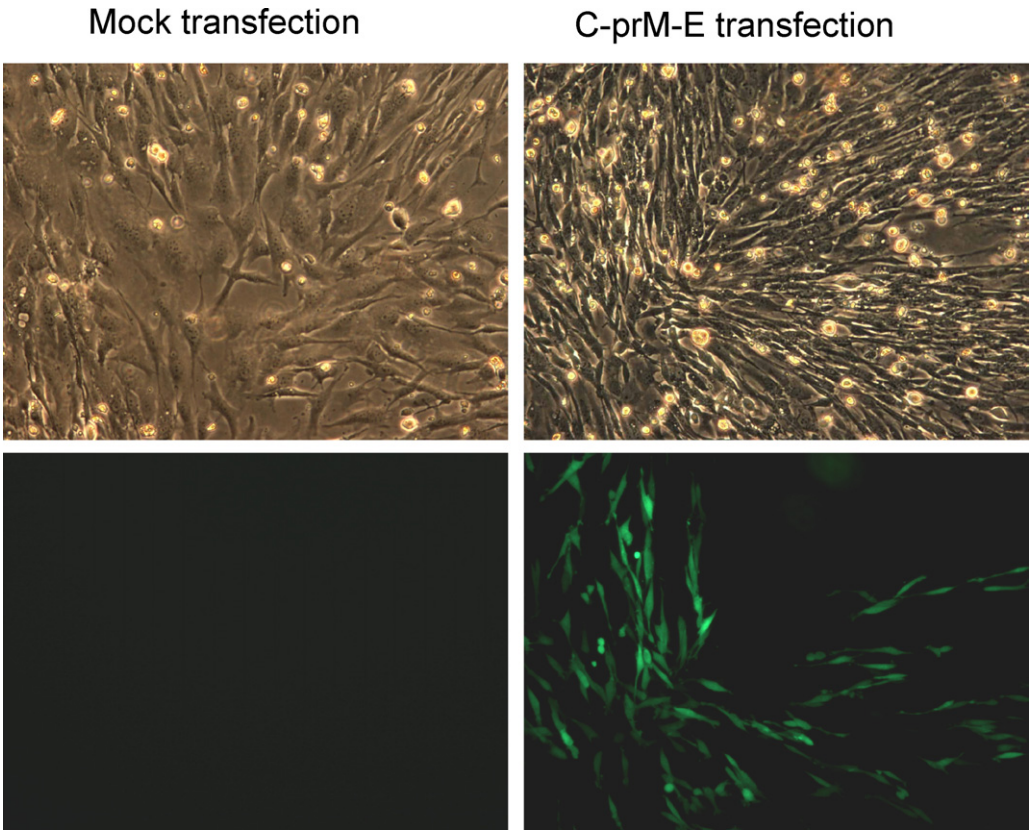


Fig. 4. EGFP-replicon packaging by C-prM-E. Top panels showed passage 6 EGFP-replicon containing cells that were either transfected with empty plasmid pXJ (Mock transfection) or with the recombinant plasmid pXJ-C-prM-E (C-prM-E transfection). The culture supernatant was collected 48 h post-transfection and 1 ml of the supernatant was collected and overlayed onto fresh BHK cells (bottom panel). EGFP expression was observed after 3 days (bottom panel).

(Fig. 4). Since, we did not obtain a large number of EGFP positive clones in the second round of infection, it can be assumed that the whole process was rather inefficient. This could be due to a low efficiency of transfection of the C-prM-E, or low packaging efficiency. At this stage we did not further evaluate the packaging efficiency or the titer of packaged viral particle. Taken together, these results showed the successfully establishment of NGC subgenomic replicon containing cell lines in which the dengue replicons replicate actively and express non-structural proteins. By expressing structural proteins *in-trans*, functional viral particles could be produced.

3.3. Inhibition of the replicon replication by a specific inhibitor and siRNA

To assess the functionality of the replicon, we incubated EGFP-replicon cells (#2.6) and Rluc-replicon cells (#29) with 2′-*C*-methyladenosine, a compound reported to inhibit both HCV and dengue RNA dependent RNA polymerase (Eldrup et al., 2004). After 72 h incubation with 50 μM of 2′-*C*-methyladenosine, the replication of the replicon as determined by the fluorescent microscopy of EGFP and NS3 decreased dramatically (Fig. 5A). This result showed that the replication of the replicon was responsive to the NS5 inhibitor and could be inhibited by the NS5 inhibitor. We proceeded to further determine the dose response study using both the EGFP- and Rluc-replicon cells. A range of 2′-*C*-methyladenosine concentrations was used in the replicon assay, and an EC_{50} curve was established 48 h post-compound incubation and calculated to be 3.6 μM in EGFP-replicon cells (#2.6) and 6.1 μM in Rluc-replicon cells (#29) (Fig. 5B and C). Employing the Rluc-replicon cells, the cytotoxicity of the compound was measured by CellTiter 96® (Promega) and no toxicity was found within the range of the concentration used up to 50 μM (Fig. 5D). This result showed that the replicon assay could be developed for quantitative measurement of the replication efficiency. Performing various experiments using Rluc-replicon cells (#29) of different passages, we determined the EC_{50} of the nucleoside inhibitor 2′-*C*-methyladenosine to be 2.42 ± 0.59 μM (Fig. 5E), with cytotoxicity of greater than 50 μM. The EC_{50} in replicon is consistent with the EC_{50} of the same compound in a whole viral infection assay in BHK cells using immunodetection of the dengue envelope protein (3.34 μM) (unpublished data, personal communication from Q.Y. Wang), indicating that the replicon assay is comparable to the whole virus infection assay for antiviral compound testing.

We have tested a series of known antiviral compounds such as ribavirin and mycophenolic acid in Rluc-replicon cells (#29). As shown in Fig. 5E, both ribavirin and mycophenolic acid showed antiviral activities ($EC_{50} = 6.77 \pm 1.33$ and 1.31 ± 0.27 μM, respectively). Chloroquine was ineffective ($EC_{50} > 12.5$ μM, data not shown), as expected from the mode of action of chloroquine in neutralizing endosomal pH in blocking viral entry, a step which is absent in the replicon.

Both EGFP- and Rluc-replicon cells were maintained in culture for more than 10 passages without significant alteration of the reporter signal. Fig. 5F shows the luciferase signal level, signal to noise ratio and the EC_{50} value for 2′-*C*-methyladenosine at passages 10, 13 and 15 in Rluc-replicon cells. Up to passage 13, the EC_{50} and signal were stable. At passage 15, a decrease in the signal was observed with a corresponding slight increase in the EC_{50} value.

In parallel with compound treatment, we have also tested siRNA designed against NS3 and EGFP in the EGFP-replicon cells (#2.6). Both siRNAs could significantly reduced the replicon signal at 3 and 4 days post-transfection, whereas a non-related siRNA (RISC-free siRNA) did not show any inhibitory effect (Fig. 5G).

4. Discussion

We describe in this paper the construction of a subgenomic replicon derived from the dengue virus type 2 strain NGC, which expresses either an EGFP or a Rluc reporter gene and a puromycin resistance gene. Employing puromycin selection, we established BHK21 cell lines stably harboring the replicon. Analysis of the puromycin resistant, EGFP- and Rluc-replicon containing BHK-21 cells revealed that the replicon encoded the viral non-structural proteins and produced RNA ensuring continuous replication of the replicon. Replicon RNA could be packaged in cells transiently expressing the C-prM-E genes *in-trans*, resulting in the formation of an "infectious" viral particle capable of a single round of infection. The BHK cell lines that harbor the replicon are stable for more than 10 passages and a nucleoside inhibitor known to inhibit the dengue NS5 polymerase inhibited the EGFP- and Rluc-replicon signal. The EC_{50} of the inhibitor measured in the replicon system is in a similar range to that measured in a full virus infection assay, showing that the replicon could closely represent viral replication. Both ribavirin and mycophenolic acid showed sub-optimal potency antiviral activities (micromolar values in EC_{50}) in the replicon system. siRNA against

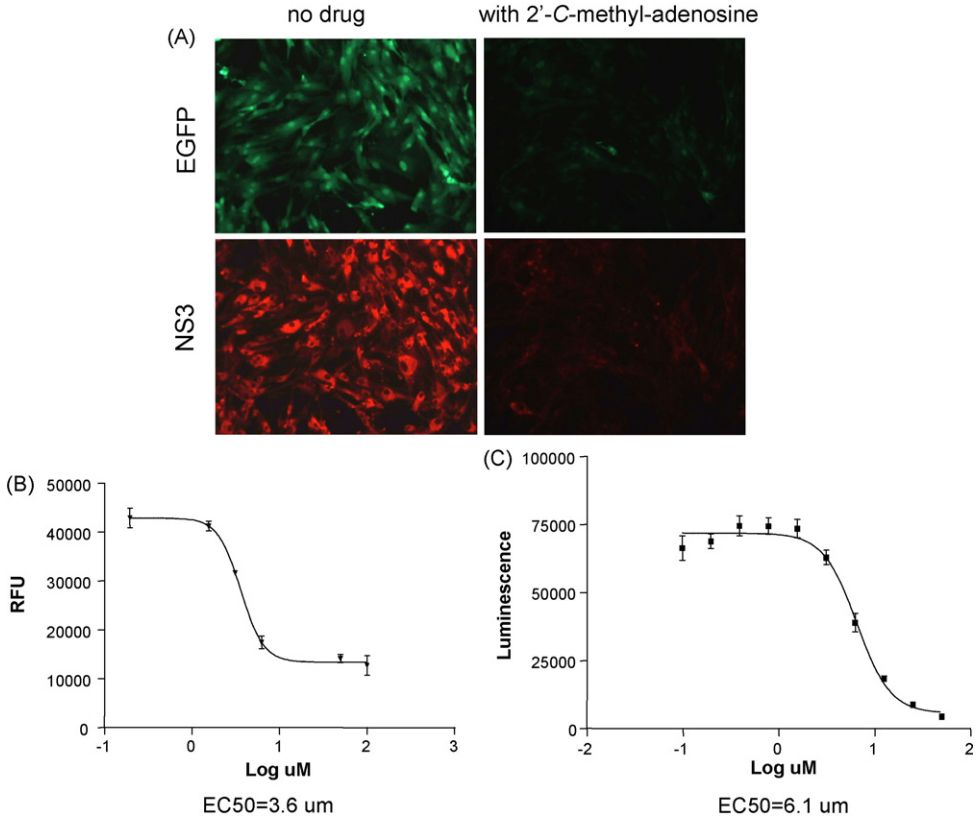
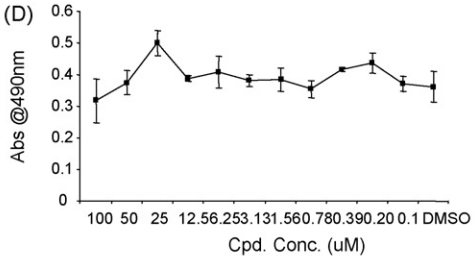


Fig. 5. (A) EGFP-replicon containing cells were incubated with 50 μM 2′-*C*-methyladenosine in 1% DMSO or with 1% DMSO alone (no drug) for 72 h in the absence of puromycin. EGFP was shown in green and NS3 was labeled in red. (B) A dose–response curve was done with 2′-*C*-methyladenosine for a range of concentrations (starting at 100 μM with a 2× serial dilution to 0.195 uM) in clone #2.6 of EGFP-replicon cells. Relative fluorescence unit (RFU) was obtained 48 h after compound addition. Data was plotted with Prism software using a sigmoidal curve fitting with variable slope. Each point represents the mean of triplicate values with error bars showing standard deviations. (C) As in (B), a dose–response curve performed with the same compound for the same range of concentration in clone #29 of Rluc-replicon cells. Luminescence was measured and data plotted as in (B). (D) As in (C), for the same range of compound concentration, cell viability was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay. (E) As in (C) and (D), the EC_{50} and CC_{50} values of 2′-*C*-methyladenosine, ribavirin and mycophenolic acid were measured in #29 Rluc-replicon cells and calculated from an average of THREE experiments. (F) EC_{50} of 2′-*C*-methyladenosine, Rluc-reporter signal (relative luminescence signal for 1×10^4 cells) and signal to noise ratio (relative luminescence signal for 1×10^4 replicon cells over 1×10^4 wild type BHK cells) were recorded for passages 10, 13 and 15 of #29 Rluc-replicon cells (P10, P13 and P15). (G) The EGFP-replicon cell #2.6 was transfected with RISC free siRNA (non-related siRNA control), EGFP siRNA or NS3 siRNA. Three or 4 days post-transfection, EGFP fluorescent signals were measured and plotted as relative fluorescent units (RFU). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

(D)



(E)

	Average EC_{50} (μM)	Average CC_{50} (μM)
2′-*C*-Methyl-adenosine	2.42 ± 0.59	>50
Ribavirin	6.77 ± 1.33	>50
Mycophenolic acid	1.31 ± 0.27	>12.5

(F)

2′-*C*-Methyl-adenosine	P10	P13	P15
EC50(μM)	2.39	2.44	3.5
Rluc signal	24056	23042	7195
Signal/noise (ratio)	43.5	40.8	16.2

(G)

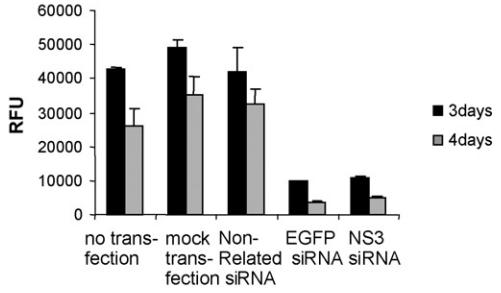


Fig. 5. (*Continued*).

NS3 and EGFP was shown to significantly reduce the replicon signal.

Among the flaviviruses, Kunjin, yellow fever and West Nile virus subgenomic replicons are well established. In dengue, several studies have reported the development of replicon systems. The very early report of the NGC subgenomic replicon (Pang et al., 2001a, b) in monkey LLC-MK2 cells was the first proof-of-concept of the self-replicating dengue subgenomic replicon, even though a very low percentage of cells (∼1%) expressed the replicon and no stable cell line was generated. In this replicon, the structural genes were deleted and replaced by a GFP reporter, with no further modification and no selection gene was inserted. Subsequent years have seen the generation of different versions of dengue replicon by several groups. Of these, Holden et al. (2006) employed the dengue 2 16681 strain as backbone in this replicon with the structural genes replaced by a firefly luciferase reporter followed by an FMDA 2A cleavage site. An antisense Hepatitis delta virus ribozyme (HDVr) sequence was engineered immediate after the 3′UTR allowing the generation of a precise 3′-end. This replicon in BHK cells showed a first peak of luciferase activity around 8 h post-transfection reflecting good translation followed by a second peak between 48 and 96 h indicating good replication. The replicon was also sensitive to mycophenolic acid (no EC_{50} value reported). Alvarez et al. (2005) generated at the same time a similar replicon from the same backbone and showed that FMDV2A cleavage was more efficient than the dengue's own NS3 cleavage site, although no HDVr sequence was used after 3′UTR. Recently, a dengue 1 Brazilian strain DV1-BR/90 was used to create a *Renilla* luciferase-reporter based replicon. The reporter was inserted after NS5 and used a separate IRES for translation (Suzuki et al., 2007). These replicons all behaved in similar transient ways in terms of translation and replication efficiency.

The construction of our replicon was inspired by the reported dengue 2 NGC replicon stably expressed in K562 and THP-1 cells (Jones et al., 2005b). The *pac* gene was employed to express puromycin selection as in Jones et al. and positioned upstream from the NS proteins. This differs from our previous unsuccessful versions of replicon which used hygromycin resistance gene, positioned downstream of the NS proteins. We believed that engineering the selection gene upstream of NS proteins exert a stronger selection pressure for the replicon to replicate and that puromycin selection allows a better selection than hygromycin. The combination of the positioning and the choice of the resistance markers were key to making a stable replicon cell line in this case. In the choice of reporter gene, we used a synthetic *Renilla* luciferase gene from Promega which optimized all low frequency codons in the original *Renilla* luciferase. This improved the efficiency of translation of the Rluc in mammalian cells and gave a better activity to score the replication. The usage of the FMDV2A cleavage site to allow the separation of the selection gene from the NS proteins was similar to most of the

reported replicons. We separated the Rluc reporter gene from the rest of the NS proteins by using a separate IRES for the genes encoding the NS proteins. For the 3′UTR, we have engineered an HDVr sequence to yield a sharp ending, though this did not differ greatly from employing the restriction enzyme XbaI cleavage, leaving an overhang of two extra nucleotides (AG). Our replicon is stable for up to passage 13 without a significant drop of the reporter signal which offers a reasonable period of time to be used for different study purpose. The comparison with other previously reported replicon could not be drawn because no information was reported on the precise duration of the reporter signal in the other reported stable cell lines.

Similar to other flavivirus replicons, the dengue replicon can be used in classical studies of viral replication, trans-complementation, viral assembly and packaging. Moreover, the stable replicon containing cell line is also a useful assay system for testing and characterization of anti-dengue compounds and studying the drug resistance profile as well as the isolation of "escape mutants" aiding the identification of an antiviral target.

Acknowledgments

We would like to thank T. Tolfvenstam (K.I.) for supervision of C.Y. Ng. C.Y. Ng is supported by an NITD graduate scholarship. Lu Thong Beng (NUS) and Mary Ng (NUS) for electron microscopy, Indira Umareddy for NS4B antibody, Aruna Sampath for NS2B-NS3 antibody. Sandra Bamberg for pXJ-C-prM-E construction, Qin Ying Wang for whole virus infection assay and Jeyaraj Duraiswamy for the chemical synthesis.

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