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Characterization of an efficient dengue virus replicon for development of assays of discovery of small molecules against dengue virus

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ABSTRACT

Dengue virus (DENV) is a public health threat to approximately 40% of the global population. At present, neither licensed vaccines nor effective therapies exist, and the mechanism of viral RNA replication is not well understood. Here, we report the development of efficient *Renilla* luciferase reporter-based DENV replicons that contain the full-length capsid sequence for transient and stable DENV RNA replication. A comparison of the transient and stable expression of this RNA-launched replicon to replicons containing various deletions revealed dengue replicon containing entire mature capsid RNA element has higher replicon activity. An efficient DNA-launched DENV replicon, pCMV-DV2Rep, containing a full-length capsid sequence, was created and successfully applied to evaluate the potency of known DENV inhibitors. Stable cell lines harboring the DENV replicon were easily established by transfecting pCMV-DV2Rep into BHK21 cells. Steady and high replicon reporter signals were observed in the stable DENV replicon cells, even after 30 passages. The stable DENV replicon cells were successfully used to determine the potency of known DENV inhibitors. A high-throughput screening assay based on stable DENV replicon cells was evaluated and shown to have an excellent *Z′* factor of 0.74. Altogether, the development of our efficient DENV replicon system will facilitate the study of virus replication and the discovery of antiviral compounds.
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1. Introduction

The four serotypes of dengue virus (DENV1–4) belong to the *Flaviviridae* family. The *Flaviviridae* family is a group of enveloped RNA viruses that includes the *Hepacivirus*, *Flavivirus* and *Pestivirus* genera. The *Flavivirus* genus consists of arthropod-borne disease agents such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and DENV (Calisher et al., 1989; Chambers et al., 1990). Many members of the *Flavivirus* genus are significant human pathogens. DENV is a public health threat to the estimated 2.5 billion people living in areas where DENV is epidemic (Guzman and Kouri, 2002, 2004; Pinheiro and Corber, 1997) and infects 50–100 million humans each year in the tropical and sub-tropical regions of the world (Noble et al., 2010). DENV infection leads to a broad spectrum of clinical symptoms, which range from being fully asymptomatic to causing life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Gubler, 1989; Halstead, 1980; Lescar et al., 2008; Pinheiro, 1989). Approximately 500,000 cases of DHF and DSS are reported in over 100 countries annually, and DENV causes approximately 25,000 deaths worldwide annually (Tomlinson et al., 2009).

DENV is an enveloped RNA virus that consists of a 10.7-kilobase single-stranded, positive-polarity genomic RNA that is associated with multiple copies of the capsid protein. It encodes one open reading frame (ORF) and translates as a single polyprotein upon entering the host cell, which is then cleaved co- and post-translationally into three structural proteins (C, M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) to enable viral replication (Chambers et al., 1990; Falgout et al., 1991; Rice et al., 1985). The ORF is flanked by the 5′- and 3′-untranslated regions (UTR), which contain several *cis*-acting RNA elements that are essential for genomic circularization and recognition of the replication complex before initiation of translation and genome replication (Lindenbach et al., 2007).

Some known *cis*-acting RNA elements in flaviviruses are located in either the 5′- or 3′-UTR (Alvarez et al., 2005a, 2008; Chiu et al., 2005; Holden and Harris, 2004; Men et al., 1996; Tilgner et al., 2005; You et al., 2001, 2009). However, critical *cis*-acting RNA elements have been discovered within the protein-coding sequences (Paranjape and Harris, 2010). For example, the cyclization sequences (CS) are present in the capsid-coding region in the 5′

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end (5′ CS) and the 3′ UTR (3′ CS) of all mosquito-borne flavivirus genomes (Alvarez et al., 2005a,b; Hahn et al., 1987; Khromykh et al., 2001; Polacek et al., 2009; Villordo and Gamarnik, 2009). Additionally, it has been reported that a hairpin element, cHP, which is located in the N-terminus of the capsid-coding region, directs start codon selection and recruits factors associated with the replicase machinery during viral RNA replication (Clyde et al., 2008; Clyde and Harris, 2006). Recently, another motif, the 5′ downstream AUG region (5′ DAR), which is located downstream of the AUG region of core gene and is complementary to the 3′ end of dengue genome, was demonstrated to be essential for RNA replication and involved in genome circularization of DENV and WNV (Fayzulin et al., 2006; Friebe and Harris, 2010; Friebe et al., 2011; Groat-Carmona et al., 2012). These results indicated that *cis*-acting elements within the flavivirus capsid gene play crucial roles in viral replication (Fayzulin et al., 2006; Friebe et al., 2012; Groat-Carmona et al., 2012).

Replicons are subgenomic, self-replicating RNA molecules that contain all of the nucleotide sequences, including the *cis*-acting elements in the 5′ and 3′ ends and the non-structural viral proteins that are required for RNA replication and translation (Moradpour et al., 2007). The establishment of an efficient hepatitis C virus (HCV), a member of the *Flaviviridae* family, replicon in cell culture systems (Blight et al., 2000; Lohmann et al., 1999; Rice, 2011) led to rapid development and discovery of anti-HCV drugs (Rice, 2011) although HCV virus replicate poorly in cell cultures whereas DENV replicates to a much higher degree. It has been demonstrated that the HCV replicon is an important tool for evaluating the potency of HCV inhibitors, and the replicon was successfully used in a broad-based proprietary antiviral screening platform. In contrast, the pace of DENV replicon development is behind that of HCV replicon development. A DENV replicon was successfully cloned (Pang et al., 2001), and its replication lasted for approximately 8 days, which is likely due to a lack of drug selection. Such a replicon design is not suitable for drug screening purposes since the cells cannot harbor DENV replicon stably. Another DENV replicon design was reported in which the DENV viral proteins were inserted downstream of the EMCV internal ribosome entry site (IRES) element and the reporter (*Renilla* luciferase)-selection marker (Pac gene) fusion DNA fragment was inserted upstream of the IRES and downstream of the 5′ UTR of the DENV genome (Ng et al., 2007). The stable DENV replicon cell lines derived from this replicon design lasted for approximately 13 passages with steady replication capacity. It implied that there is an issue regarding the stability of replicon replication in the two above DENV replicons during the passages of DENV replicon cells unlike the stable HCV replicon cells, which have been shown to replicate steadily even after one year passage of cells (Pietschmann et al., 2001). Recently, a monocistronic design of RNA-launched DENV replicon with EGFP reporter was reported (Masse et al., 2010). DENV replicon cells were therefore established and applied to screen inhibitors by HTS assay with good *Z*′ factor (>0.7) and signal-to-noise ratio (20–25) (Masse et al., 2010). The other DNA-launched dicistronic DENV GFP reporter replicon was successfully shown to develop stable DENV replicon cells for screening antiviral compounds (Leardkamolkarn and Sirigulpanit, 2012; Leardkamolkarn et al., 2012). In general, the DENV GFP stable cell clones steadily expressed between passage 10 and 30. Another monocistronic DENV replicon with luciferase reporter was designed and stable replicon cells were created for HTS of DENV inhibitors with a good *Z*′ factor (0.62) (Hsu et al., 2012) although there is no information regarding the expression of DENV replicon cells during passage of cells.

In this study, we established efficient RNA and DNA-launched DENV reporter replicons that are capable of transiently and stably expressing foreign and viral proteins in host cells. We accomplished this by mapping the essential elements within the capsid-coding region of DENV. We found that the replicon containing a full-length functional capsid gene (nts 97–402) has the highest replication efficiency. We further established single colonial stable replicon cell lines harboring the DNA-launched DENV reporter replicon, which can be used for antiviral compound screening and evaluation. There was no apparent difference in the luciferase activity over 30 passages of the DENV replicon containing cells. These findings may provide a system that will permit further understanding of the role of the capsid-coding region in *flavivirus* RNA replication and the development of a useful, efficient and long-lasting replicon system that can be used for drug discovery or vaccine development.

2. Materials and methods

2.1. Cell lines

The baby hamster kidney (BHK21) cells (ATCC CCL-10) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) that was supplemented with 4.5 g/L glucose and 2% fetal bovine serum (FBS). The Huh7.5 cells were grown in Dulbecco's minimal essential medium (DMEM) that was supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS, Gibco), and non-essential amino acids (NEAA, Gibco). The cells were cultured at 37 °C in a 5% CO_2 incubator.

2.2. Escherichia coli and yeast strains

Frozen, competent *E. coli* strain C41, a derivative of BL21 (DE3) (Miroux and Walker, 1996), was purchased from OverExpress Inc. Standard yeast medium and transformation methods were used (Burke, 2000). *Saccharomyces cerevisiae* YPH857 was purchased from ATCC. The genotype YPH857 is MATα ade2-101 lys2-801 ura3-52 trp1-Δ63 HIS5 CAN1 his3-Δ200 leu2-Δ1 cyh2. Competent yeast cells were prepared using the lithium acetate procedure (Burke, 2000).

2.3. Construction of the RNA-launched DENV reporter replicons

All of the primers that were used for PCR synthesis are shown in the Table 1. The DENV reporter replicon (nt402) was constructed from the full-length cDNA clone of DENV strain 16681, which was kindly provided by Dr. Nopporn Sittisombut (Sriburi et al., 2001). The detailed methods for the construction are described in a previous report (Yang et al., 2011). We further constructed six truncation replicon clones, each carrying a deletion of a different size within the capsid gene of the DENV reporter replicon, nt402. First, three deletion mutants (nt306, nt207, and nt162) were generated using overlapping extension PCR and the WT as the template. Initially, the two primary fragments for overlapping PCR were amplified during the first round of PCR with the following pairs of primers: (i) nt306 PCR1: *SacI*-Sp6-5UTR-F + nt306R; nt306 PCR2: nt306F + Neo-R, (ii) nt207 PCR1: *SacI*-Sp6-5UTR-F + nt207R; nt207PCR2: nt207F + Neo-R, and (iii) nt162 PCR1: *SacI*-Sp6-5UTR-F + nt162R; nt162 PCR2: nt162F + Neo-R. For all constructs, two primary PCR products were gel purified and subsequently fused using overlapping PCR with the primers *SacI*-Sp6-5UTR-F and Neo-R. The resulting fragments were gel purified and joined using homologous recombination of the two DNA fragments, including each of the deletion mutant fragments and the linearized nt402 plasmid (digested with *SacI* and *SphI*), in competent yeast cells. Competent yeast cells were prepared using the lithium acetate method (Gietz and Woods, 2002). Cloning of the following RNA- and DNA-launched DENV reporter replicons were based entirely on homologous recombination in yeast cells;

Table 1
Oligonucleotide primers employed for cDNA synthesis and PCR amplification.

Oligonucleotides	Sequence
SacI-Sp6-5UTR-F	5′-AGTGAATTGTAATACGACTCACTATAGGGCGAATTG GAGCTCATTTAGGTGACACTATAGAGTTG-3′
C102-R	5′-GGGGTCGTACACCTTGGAAGCCAT TGCAGTTCTGCG CCTCCTGTTCAA-3′
Neo-R	5′-GAAGAACTCGTCAAGAAGGCGATA-3′
nt306R	5′-CTTGGAAGCCATGGCAGATCTGCGTCTCCTATT CAATCCCCATCTCTTCAATATCCCTGC-3′
nt306F	5′-ATCCCACCAACAGCAGGGATATTGAAGAGAT GGGGATTGAATAGGAGACGCAGATCTGCC-3′
nt207R	5′-CTTGGAAGCCATGGCAGATCTGCGTCTCCT ATTCAACATTCCAAGTGAGAATCTCTTTGT-3′
nt207F	5′-GTGCAACAGCTGACAAAGAGATTCTCACTT GGAATGTTGAATAGGAGACGCAGATCTGCC-3′
nt162R	5′-CTTGGAAGCCATGGCAGATCTGCGTCTCCTATT CAAGCGGTTTCTCTCGCGTTTCAGCAT-3′
nt162F	5′-ACGCCTTTCAATATGCTGAAACGCGAGAGAAA CCGCTTGAATAGGAGACGCAGATCTGCC-3′
DV2.PS-R	5′-AGAACCTGTTGATTCACAAGC-3′
DV2.L1-R	5′-CATTCCATTTTCTGGCGTTCT-3′
DV2.U2-F	5′-AAGGTGAGATGAAGCTGTAGTCTC-3′
DV2.P1	5’-CTGTCTCCTCAGCATCATTCCAGGCA-3’
IRES-F	5′-CTCAAGCGTATTCAACAAGGG-3′
pCMV-F	5′-TGTCGAGTTTACTCCCTATCAGTGATAGAGAA CGTATGTCGAGGTAGGCGTGTACGGTGG-3′
CMV-5′UTR-R	5′-GTCGGTCCACGTAGACTAACAACTGACGGTT CACTAAACGAGCTCTGC-3′
CMV-5′UTR-F	5′-GCAGAGCTCGTTTAGTGAACCGTCAGTTGTTAGTCTACGTGGACCGAC-3′
3UTR-F	5′-GAAGCAGGAGTTCTGTGGTAGAAAGC-3′
HDVr-R	5′-GGGCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGT CCCGCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCGGATGCCCAGGTCGGA CCGCGAGGAGGTGGAGATGCCATGCCGACCCAGAACCTGTTGATTCAACAGCACCATT CCATTTTCTG-3′

recombinant DNA *in vitro* ligation was not used. Successful recombination in yeast cells requires the termini of the two recombinant DNAs, which are to be joined together, to contain 40 base pairs or more of homologous sequences. Finally, the truncation replicon clones were purified from yeast cells, re-amplified in *E. coli* strain C41 and verified by DNA sequencing.

2.4. RNA transcription and transient replication activity assay of the RNA-launched DENV replicon

cDNA plasmid of replicon was linearized with *XbaI*. DNA was phenol–chloroform extracted, precipitated and used as a template for *in vitro* transcription using a SP6 Message mMachine kit (Ambion). The RNA was quantified by spectrophotometer and stored at −80 °C. The transient replicon assay was performed to quantify the effects of deletions within the capsid-coding region on viral RNA translation and replication (Friebe et al., 2012). The variant RNA transcripts were transfected into BHK21 or Huh7.5 cells by electroporation. Briefly, subconfluent BHK21 or Huh7.5 cells were detached by using trypsin, collected by centrifugation (500*g*, 5 min), washed three times with 4 °C sterile phosphate-buffered saline (PBS), resuspended 2×10^6 cells in 400 μl electroporation buffer (Eppendorf Hypoosmolar Buffer: Eppendorf Isoosmolar Buffer = 64 μl:336 μl) and incubated for 30 min at 4 °C. RNA transcripts (1 μg) were mixed with 0.4 ml of resuspended cells in a 2-mm gap cuvette (Molecular Bioproducts) and pulsed (500 V; pulse-length, 200 μs; 2 pulses) using an Eppendorf Multiporator. Pulsed cells were left to recover for 20 min at room temperature and the diluted into 10 ml culture medium. Cells were plated in 10 wells of 12-well plate.

To perform the luciferase assays, at each time point the medium was removed and the cells were washed with PBS, lysed by adding 100 μl of lysis buffer (Promega, Renilla luciferase assay system), and then assayed according to the manufacturer's protocol (Promega, Renilla luciferase assay system). Duplicated wells were lysed at the times indicated and measured using a GloMAX 20/20 Luminometer (Promega). To quantify the replication efficiency, the relative luciferase activity was normalized to the signal at 4 h post-transfection.

2.5. Immunofluorescence analyses

For the detection of transient expression of DENV replicon, BHK21 cells (2×10^5 and 3×10^4 for electroporation and transfection, respectively) for immunofluorescence analyses were either electroporated with 100 ng nt402 replicon RNA for 48 h or transfected with 100 ng pCMV-DV2Rep plasmid for 72 h. The replication-defective replicons, sp6-NS5 mut and NS5 mut, were also side by side used as negative control. For detection of replicon expression within DENV replicon cells, Clone 5 DENV replicon cells (5×10^4 cells) at passage 10 (P10) or 30 (P30) were used for immunofluoroscence assay. Cells were washed once with PBS, fixed with 1% paraformaldehyde for 1 h in room temperature, permeabilized with 1% Triton X-100 in PBS for 10 min in room temperature, and blocked with 2% horse serum in PBS. Viral complexes were detected using the mouse monoclonal anti-DENV NS3 antibody (#YH0034, Yao-Hong Biotechnology Inc.). The secondary AF488 anti-mouse antibody was purchased from Invitrogen.

2.6. Western blot analyses

The cells (3×10^5 cells) were collected, washed by PBS, and then lysed in 40 μl of cold RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.2% SDS and complete Mini, EDTA-free protease inhibitor (Roche)). Cell lysate was centrifuged at 14,000*g* for 10 min at 4 °C. The supernatant was separated by 8% SDS–PAGE

electrophoresis at 100 V and then transferred to a 0.2 μm PVDF membrane (Pall Corporation) using 220 mA at 4 °C for 100 min. The membrane was blocked with nonfat dry milk (5% w/v) in TBST (Tris-buffered saline containing 0.05% Tween-20) for 1 h at room temperature, and then incubated with mouse monoclonal anti-DENV NS3 antibody (1:1000 dilution in TBST) (#YH0034, Yao-Hong Biotechnology Inc.) or with rabbit polyclonal anti-GAPDH antibody (1:3000 dilution in TBST) (#GTX100118, GeneTex, Inc.), overnight at 4 °C. After washing, the membranes were incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG polyclonal antibody (GeneTex, Inc.). The enzyme reaction was developed using chemiluminescent HRP substrate (Immobilon ™Western, #WBKLS0500, Millipore Corporation).

2.7. Quantitative RT-PCR

Real-time PCR was performed as described previously (Yang et al., 2011). Briefly, the viral RNA in the transfected BHK21 or Huh7.5 cells was isolated using the Qiagen RNeasy Kit as described in the manufacturer's protocol. Viral RNA was reverse transcribed to cDNA using the Invitrogen Thermoscript RT Kit with specific primer (DV2.PS-R) for detection of positive-sense viral RNA based on the manufacturer's protocol. Real-time quantitative PCR was used primer pair (DV2.U2-F and DV2.L1-R) and hydrolysis probe DV2.P1 to measure the viral RNA (Yang et al., 2011). The LightCycler TaqMan Master Kit (Roche) and LightCycler 1.5 Instrument (Roche) were utilized in this study under the following thermocycling conditions: pre-incubation at 95 °C for 10 min followed by 45 cycles of three-step incubations at 95 °C for 15 s (denaturation), 60 °C for 30 s (annealing and elongation), and 72 °C for 1 s (complete elongation with a single fluorescence measurement). A linear relationship was established between the RNA copy number per milliliter and the corresponding threshold cycle (C_T) value over seven logs of RNA concentration (correlation coefficient, $r = 0.99$) (Houng et al., 2000).

2.8. Selection of a single colonial RNA-launched replicon cell line

BHK21 cells (1×10^4 per well in a 24-well tissue culture plate) were plated for 16 h, and then transfected with 0.5 μg of nt207 or nt402 replicon RNAs using 1.5 μl of Lipofectamine™ 2000 (Invitrogen). The next day, transfected cells were trypsin digested and diluted with culture medium containing 1 mg/ml G418 (Amresco) to a final concentration of 20 cells/ml. Finally, we added 100 μl of diluted cells to a 96-well culture plate and replaced the medium with fresh G418 medium every three days. After 10–14 days, when the clones were visible by eye, clones were individually picked and amplified for the luciferase assay.

2.9. Construction of the DNA-launched DENV reporter replicon

All of the primers used for the following PCR synthesis are shown in the Table 1. To construct the DNA-launched reporter replicon, pCMV-DV2Rep, the fragments 5′UTR-C102-Rluc-2A-Neo-IRES-E24-NS-3′UTR and 3′UTR-HDVr were generated with the following pairs of primers by extension PCR using nt402 as the template (i) CMV-5′UTR-F + DV2.L1-R for the 5′UTR-C102-Rluc-2A-Neo-IRES-E24-NS-3′UTR fragment, and (ii) 3UTR-F + HDVr-R for the 3′UTR-HDVr fragment. The pCMV-5′UTR fragment was generated with primers pCMV-F and CMV-5′UTR-R by extension PCR using pTRE-Tight (Clontech) as the template. The three PCR products were gel purified and subsequently constructed through homologous recombination of the four DNA fragments, which included pCMV-5′UTR, 5′UTR-C102-Rluc-2A-Neo-IRES-E24-NS-3′UTR, 3′UTR-HDVr and linearized nt402 plasmid (digested with *SacI* and *XbaI*), in yeast cells. Competent yeast cells were prepared using the lithium acetate method (Gietz and Woods, 2002). Finally, the DNA-launched replicon clones were purified from yeast cells, re-amplified in *E. coli* strain C41 and verified by DNA sequencing.

2.10. Transient replication activity assay of the DNA-launched DENV replicon

BHK21 cells were plated at a density of 1×10^4 per well in a 24-well tissue culture plate and incubated for 16 h at 37 °C. Fifty nanograms of the DNA-launched replicon pCMV-DV2Rep were transfected into one well with 0.25 μl of Lipofectamine™ 2000 (Invitrogen). To perform the luciferase assays, at each time point (24, 48, 72, and 96 h post-transfection), the medium was removed, and the cells were washed with PBS, lysed by adding 100 μl of lysis buffer (Promega, Renilla luciferase assay system), and then assayed according to the manufacturer's protocol (Promega, Renilla luciferase assay system). Duplicated wells were lysed at the times indicated and measured using a GloMAX 20/20 Luminometer (Promega). To quantify the replication efficiency, the relative luciferase activity was normalized to the signal at 24 h post-transfection. The luminescence reading at 72 h post-transfection was plotted against the log transformation of the concentration of the compound and a sigmoidal curve fit with variable slope was created to determine the 50% inhibitory concentration (IC_{50}) value using Prism v.5 software (Graphpad software, San Diego, CA). Each dataset was fitted separately. The results represent the means ± the standard errors of the mean (SEM) of duplicate determinations from three independent experiments.

2.11. Cytotoxicity assay

The sensitivity of the cell lines to the compounds was examined using the MTS-based tetrazolium reduction assay CellTiter 96 AQueous Non-Radioactive cell proliferation assay (Promega G5430). Briefly, DENV replicon cells were plated at a density of 1.5×10^4 cells per well in 96-well plates containing 120 μl of culture medium for 6 h. Serially diluted compounds or DMSO (positive control) were added and incubated for an additional 72 h. MTS reagent was then added to each well and incubated for 1 h at 37 °C in a humidified 5% CO_2 atmosphere before reading at a wavelength of 490 nm using an ELISA plate reader. The CC_{50} values were plotted with Prism v.5 software (Graphpad software, San Diego, CA) using a sigmoidal curve fitting with a variable slope. Each dataset was fitted separately. All data are presented as the means ± SEM from three independent experiments.

2.12. Selection of single colonial DNA-launched replicon cell lines

BHK21 cells were plated at a density of 1×10^4 per well in a 24-well tissue culture plate and incubated for 16 h at 37 °C. BHK21 cells were transfected with 50 ng of the pCMV-DV2Rep replicon using 0.25 μl of Lipofectamine™ 2000 (Invitrogen). The next day, the transfected cells were trypsin digested and diluted with culture medium (DMEM containing 2% FBS) containing 1 mg/ml G418 to a final concentration of 20 cells/ml. Finally, we added 100 μl of diluted cells into each well of a 96-well culture plate and replaced the medium with fresh G418 containing medium every three days. After 10–14 days, when clones were visible by eye, six clones were individually picked and amplified for stock and for the luciferase assay.

Each clone (3×10^4 per well) was seeded in 24-well plates for 72 h, and then subcultured for next passage. To perform the luciferase assays of each passage, 1×10^5 cells were lysed by adding 100 μl of lysis buffer (Promega, Renilla luciferase assay system), and then assayed according to the manufacturer's protocol (Promega, Renilla luciferase assay system). Duplicated cells were lysed and measured

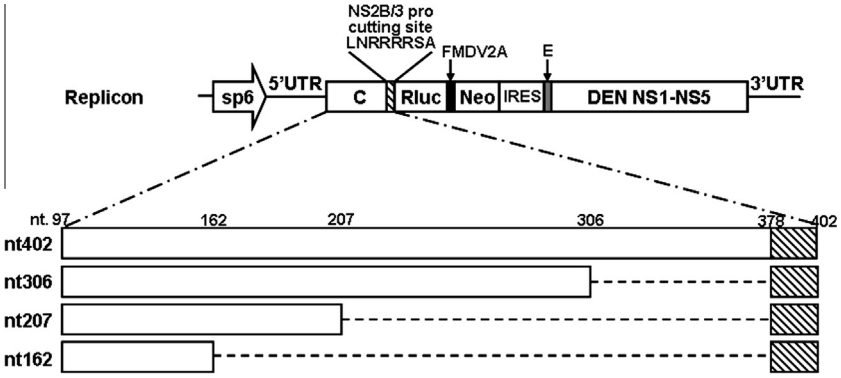


Fig. 1. Schematic representation of the DENV capsid deletion mutants that were constructed in the context of a subgenomic DENV reporter replicon. Shown at the top is the construction of the DENV strain 16681 reporter replicon. The 5′-UTR, the coding sequence of the Capsid protein (C), the Renilla luciferase gene (Rluc), the FMDV2A cleavage site (black box), the neomycin resistance gene (Neo), the EMCV IRES element (gray box), the C-terminal 24 amino acids of the E protein (E), the entire NS regions (NS1–NS5), and the 3′-UTR are indicated. The slash box indicates the NS2B/3 pro recognition site that is used for proteolytic digestion. The three capsid protein deletion clones are illustrated above. The nucleotides are numbered from the first base of the 5′-UTR. The nucleotides present in each construct are indicated by the name assigned to it. The regions that were deleted from each of the individual constructs are shown by dotted lines. These replicons were used to determine sequences required for viral RNA replication of DENV.

using a GloMAX 20/20 Luminometer (Promega). The procedure was repeated for 34 passages.

2.13. Compound susceptibility assay using the DENV replicon cells

Clone 5 DENV replicon cells were plated at a density of 1×10^4 per well in a 24-well tissue culture plate. The next day, the culture medium (DMEM containing 2% FBS and 1 mg/ml G418) was replaced with DMEM containing 2% FBS and serial diluted compound. After 48 h of treatment, the culture medium was removed and the cells were washed with PBS, lysed with 100 μl of lysis buffer (Promega), and then assayed according to the manufacturer's protocol (Promega). Duplicated wells were lysed at the times indicated and measured using a GloMAX 20/20 Luminometer (Promega). The luminescence signal was plotted against the log transformation of the concentration of the compound and a sigmoidal curve fit with variable slope was created to obtain the 50% inhibitory concentration (IC_{50}) value using Prism v.5 software (Graphpad software, San Diego, CA). The results represent the means ± standard error of the mean (SEM) from duplicate determinations from three independent experiments.

2.14. Characterization of the 30th passage (P30) of Clone 5 DENV replicon cells

The 30th passage (P30) of Clone 5 DENV replicon cells (5×10^5 cells) were collected and extracted total RNAs using the Qiagen RNeasy Kit as described in manufacturer's protocol. Viral RNAs were amplified by RT-PCR using primer pair (IRES-F and DV2.L1-R) and SuperScript III one-step RT-PCR kits (Invitrogene). The PCR products of NS1–NS5 were gel-purified and subcloned into the shuttle vector pRS313 (Sikorski and Hieter, 1989) through homologous recombination in yeast cells. The plasmids were purified from yeast cells and re-amplified in *E. coli* strain C41 for DNA sequencing.

2.15. Pilot high-throughput screening (HTS) by using DENV replicon cells

The HTS was performed at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan. The compound libraries used in this primary screen were purchased from Chemical Diversity Lab (San Diego, CA) and had >95% purity. Compounds from a compound library with diverse structures were provided as DMSO stock solutions at 10 mM. Eight thousand compounds were screened in the pilot screen. The selected clone of the reporter replicon cells was plated at a density of 4×10^3 cells per well in 96-well plates. The next day, the stable replicon cells were treated with 10 μM of the various compounds or DMSO as a control. 15 μM of BP2109 were also tested in each plate as reference inhibitor. After a 24 h incubation, the compound-treated cells were washed with PBS and lysed with 20 μl/well of 1× lysis buffer (Promega, *Renilla* luciferase assay system E2820), and the luminescence was measured using the Paradigm Luminescence.

2.16. Z′ factor calculation

The Z′ factor was calculated to evaluate the performance of the HTS assay. It is defined as follows: Z′ factor = 1-[(3SD of signal + 3SD of background)/|(mean of signal − mean of background)|]), where SD is the standard deviation (Zhang et al., 1999). A larger Z′ factor indicates a higher suitability of the assay for HTS (Zhang et al., 1999). The Z′ factor of each screen was calculated and plotted with a mean of 0.74 and a SD of 0.05, which indicates that our HTS with the DENV replicon system is an excellent assay with good separation between the background controls (Zhang et al., 1999).

2.17. Statistical analysis

Data were compared side by side with Prism v.5 software (Graphpad software, San Diego, CA). Comparisons of the nt402 replicon with the truncated mutant replicons were analyzed for significant differences using Student's *t* test with Welch's correction to measure the two-tailed *P* values. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Characterization of the various DENV mutant replicons revealed that the DENV replicon with full-length capsid RNA element has the highest transient replicon activity

To the best of our knowledge, the design of the previously reported DENV replicons primarily consists of C22–27 (the first 22–27 amino acids of the capsid gene) (Holden et al., 2006; Jones

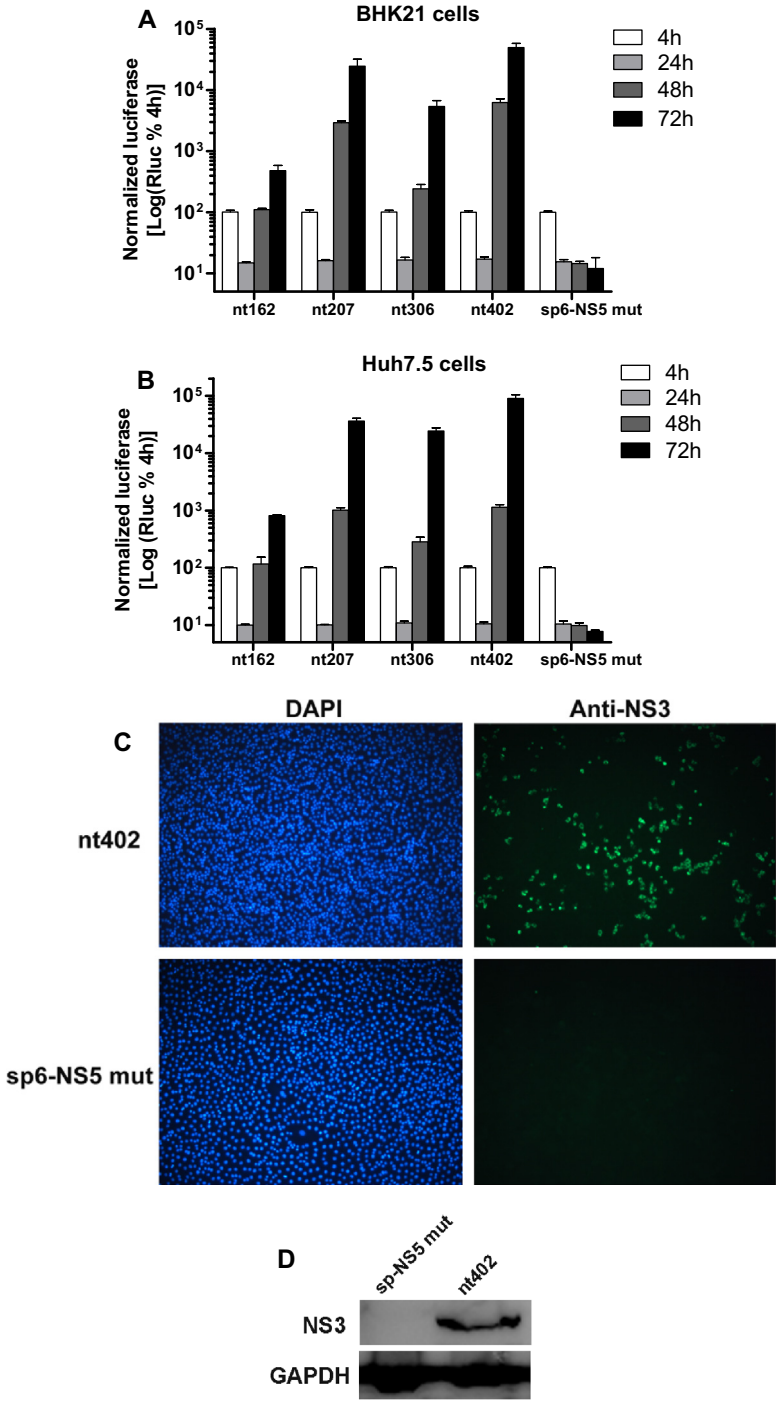


Fig. 2. DENV replicon activity is affected by deletion of the capsid gene within the replicon context. (A) The replication efficacy of the variant DENV reporter replicons in BHK21 cells. The luciferase activity in the cytoplasmic extracts prepared from BHK21 cells that had been electroporated with variant DENV replicon RNAs was measured and is shown in a logarithmic scale at the indicated time points and is normalized to the results at 4 h post-transfection. (B) Replication efficacy of the variant DENV reporter replicons in Huh7.5 cells. Luciferase levels are shown on a logarithmic scale at the indicated time points and were normalized to the results from 4 h post-transfection. The mean values and SEMs from three independent experiments are plotted. (C) Immunofluorescence assay of cells electroporated with the same amount of RNAs obtained from the nt402 or replication-defective sp-NS5 mut replicon. IFA images were taken at 100× magnification showing expression of DENV NS3 protein in electroporated cells at 48 h post-electroporation. Green cells indicate positive cells. The cells were nuclear stained with DAPI (blue color) (D) Western blot analysis of DENV NS3 protein. The expression level of NS3 protein was compared with the BHK21 cells electroporated with the same amount of RNAs obtained from the nt402 or replication-defective sp-NS5 mut replicon. GAPDH was also detected as loading control.

et al., 2005; Lee et al., 2010; Masse et al., 2010; Mosimann et al., 2010; Ng et al., 2007; Pang et al., 2001) or C34 (the first 34 amino acids of the capsid gene) (Alvarez et al., 2005a; Suzuki et al., 2007) of the capsid-coding region, which are required for virus replication. Recently, several DENV replicon designs were reported to utilize the full-length capsid gene (Hsu et al., 2012; Leardkamolkarn and Sirigulpanit, 2012; Leardkamolkarn et al., 2012; Yang et al., 2011). To further investigate the sequences within the

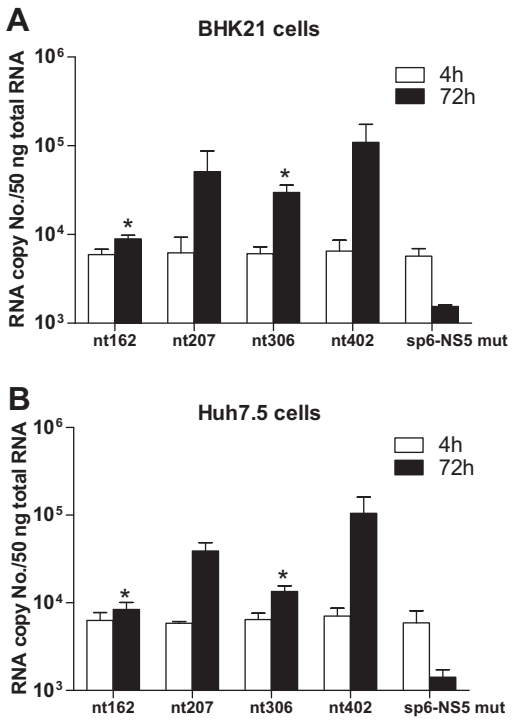


Fig. 3. RNA synthesis of DENV is affected by the deletion of the capsid gene within the DENV replicon context. (A) Deletions within the capsid-coding region affect viral RNA synthesis in BHK21 cells. The variant replicon RNAs were electroporated into BHK21 cells. The positive-strand viral RNA copy number at 4 h and 72 h post-transfection of BHK21 cell with variant DENV replicon RNAs are measured by real time RT-PCR. (B) The RNA synthesis level of the variant DENV mutant replicon RNAs in Huh7.5 cells. As described in (A), the variant replicon RNAs were electroporated into Huh7.5 cells, and the cells were collected to measure the level of RNA synthesis. The error bars represent the SEMs from three independent experiments ($n = 3$). $^*p < 0.05$.

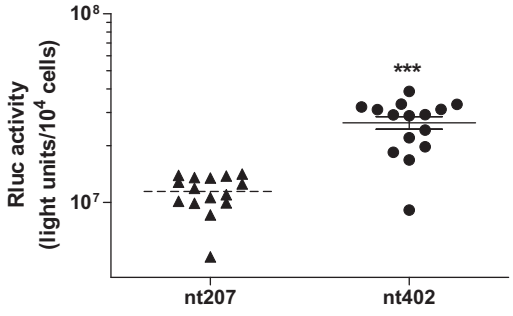
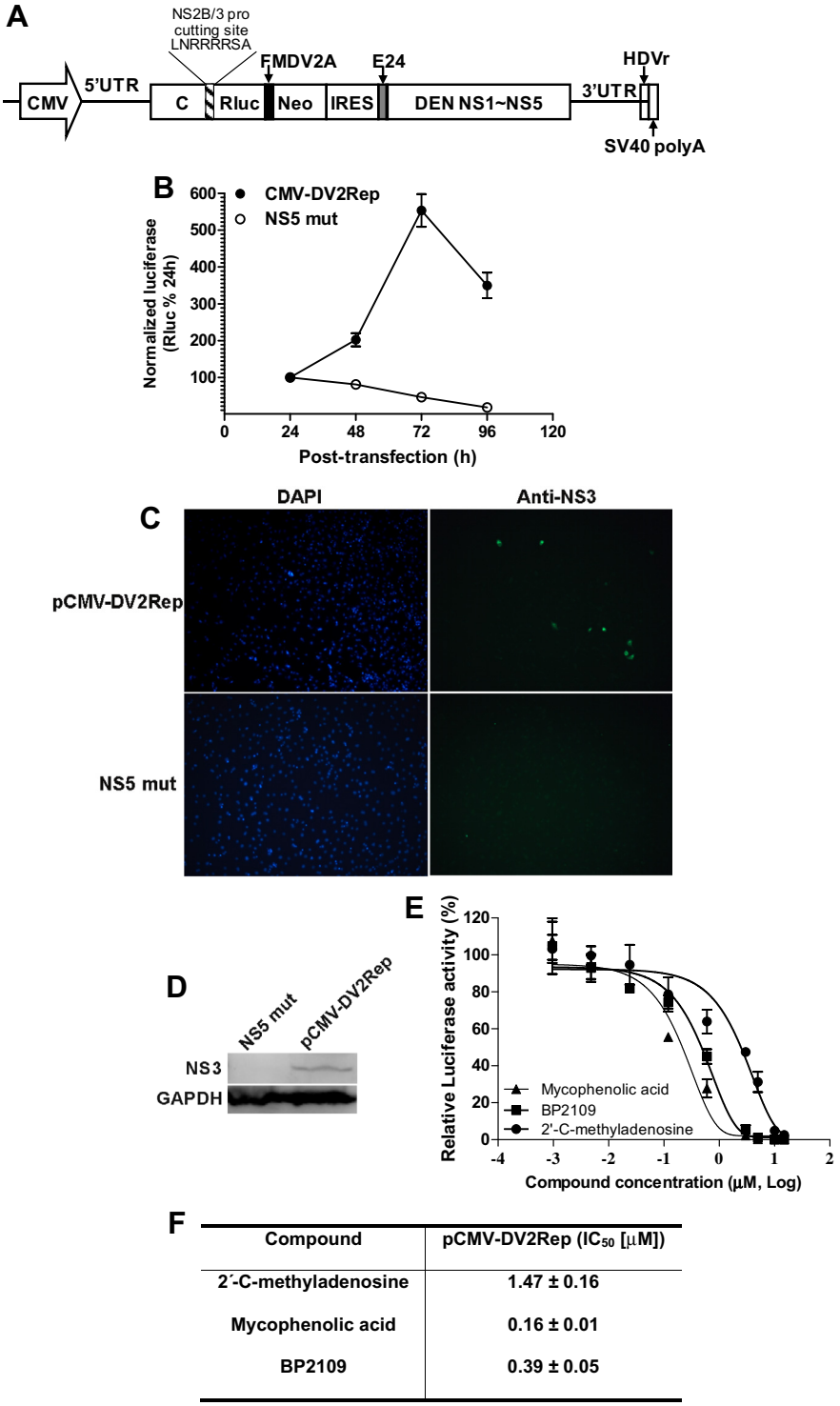


Fig. 4. The C-terminal portion of the DENV capsid-coding sequence plays a role in viral RNA replication in BHK DENV replicon cells. The nt207 and nt402 replicon transcripts were transfected into BHK21 cells and then treated with G418 (1 mg/ml) at 4 h post-transfection. After the cells had been treated with G418 for 2 weeks, the colonies were individually picked and amplified. A luciferase assay was performed to measure the luciferase levels in fifteen randomly picked colonies ($n = 15$). The broken horizontal lines show the means of the fifteen colonies. The error bars represent the SEMs. $^{***}p < 0.0001$.

capsid-coding region that are required for DENV replication, we performed truncation mutagenesis of the DENV capsid-coding region to fine map the critical regulatory RNA elements that are required for viral RNA replication. Various deletions were introduced into the full-length capsid-coding region in the context of a DENV reporter replicon, nt402, to generate a total of three mutant replicons (Fig. 1). The designation of each of the three mutants indicates the nucleotide (nt) position from the first based of the 5′-UTR present in each construct (Fig. 1). For example, the nt306 replicon contains the first 306 nts of the DENV genome. During flavivirus polyprotein processing, the capsid protein is first anchored to the ER membrane and is then digested by the viral NS2B/3 protease in the cytoplasm. To release the same amount of co-translated *Renilla* luciferase (Rluc) protein into cytoplasm, the replicons contained the NS2B/3 recognition site at the N-terminus of the Rluc protein and an artificial FMDV2A cleavage site at the C-terminus of the Rluc protein (Fig. 1).

To map potential regulatory sequences within the DENV capsid gene, we first analyzed the effect of these deletions on the replication capacity of various DENV replicon RNAs in BHK21 and Huh7.5 cells. We transfected BHK21 and Huh7.5 cells with RNA transcripts synthesized from various replicon cDNA templates and monitored the luciferase signal at different time points. The luciferase signal at 4 h reflects the translation efficiency and the transfection efficiency of the input RNAs. All signal values were normalized to the 4 h value to account for transfection efficiency. The luciferase signals at 4 h post-transfection were compared among the nt162, nt207, nt306, nt402, and sp6-NS5 mut replicon RNAs, and no apparent difference in the transfection and translation efficiency was observed among the four replicons (data not shown). To ensure that amplification of the Rluc signal was mediated through RNA replication, a replication-defective replicon was designed by mutating the GDD motif of NS5 to GAA (sp6-NS5 mut) as a negative control (Fig. 2C and D). In both the BHK21 and the Huh7.5 cells, we found that replicon nt402, which contains the entire mature capsid gene sequence, demonstrated the highest replication capacity (Fig. 2A and B). We detected the expression of DENV NS3 proteins within the cells electroporated with nt402 replicon RNAs by immunofluorescence assay (~15% of cells with positive NS3 staining) and Western blotting at 48 h post-electroporation (Fig. 2C and D). The replicon (nt207 replicon) containing nts 97–207 of the DENV capsid sequence replicates less efficiently than the nt402 replicon. The nt306 replicon, which contains nts 97–306 of the DENV capsid sequence, showed comparable replication efficiency to the nt207 replicon. Another replicon construct, nt162, which contains nts 97–162, displayed the lowest replication capacity among the deletion DENV replicon constructs. The difference in the luciferase activity between constructs nt207 and nt162 replicons was observed 48 h post-transfection in both the BHK21 (~26- and 51-fold increase at 48 h and 72 h post-transfection, respectively) (Fig. 2A) and the Huh7.5 cells (~9- and 44-fold increase at 48 h and 72 h post-transfection, respectively) (Fig. 2B). These results suggest that the RNA sequence located between nts 163–207 of the DENV genome is essential for replication of the DENV replicon. Furthermore, the nt402 replicon had ~26- and 9-fold higher replication efficacy than the nt306 replicon at 48 h and 72 h post-transfection in BHK21 cells, respectively (Fig. 2A) and an ~4-fold increase at 48 and 72 h post-transfection in the Huh7.5 cells, respectively (Fig. 2B). These results suggest that deletion of nts 307–378 of the DENV genome reduced the replication efficiency of the nt402 replicon, which indicates that nts 307–378 of the DENV genome are required for full replication efficacy.

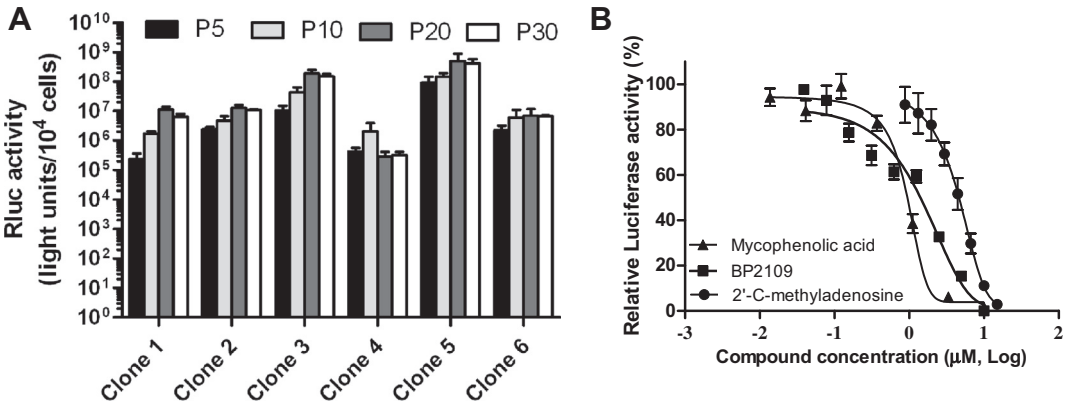
To further determine if the truncations in the DENV capsid gene sequence affected the production levels of viral RNA, DENV RNA derived from the various DENV replicon constructs was measured by quantitative RT-PCR at 4 and 72 h post-transfection. No apparent difference was observed between the viral RNA levels of the various DENV replicons at 4 h post-transfection (Fig. 3A and B), which indicates that all of the DENV replicon constructs had similar transfection efficiencies. Consistent with the results from the luciferase activity, there were also significant increases in the level of positive-stranded viral RNAs derived from the nt402 replicons compared to the nt162, nt207, and nt306 replicon at 72 h



F

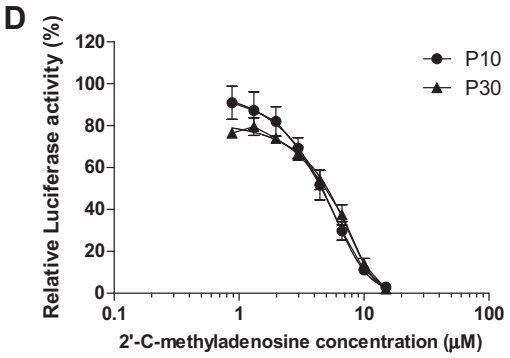
Compound	pCMV-DV2Rep (IC_{50} [μM])
2′-C-methyladenosine	1.47 ± 0.16
Mycophenolic acid	0.16 ± 0.01
BP2109	0.39 ± 0.05

Fig. 5. Characterization of transiently expressed DNA-launched DENV replicons. (A) A schematic diagram of the DNA-launched DENV replicon construct, pCMV-DV2Rep. The construct contains the cytomegalovirus (CMV) promoter, DENV sub-genomic sequences from the nt402 replicon, and the antigenomic sequences of the hepatitis delta virus ribozyme (HDVr). The HDVr sequence ensures the generation of the correct DENV 3′ terminus and the simian virus 40 (SV40) polyadenylation signal. (B) Replication efficacy of the DNA-launched DENV replicon in BHK21 cells. The pCMV-DV2Rep plasmid was transiently transfected into BHK21 cells, and the luciferase activity was monitored at the indicated time points and normalized to the results from 24 h post-transfection. The mean values and SEMs from three independent experiments are plotted. (C) Immunofluorescence assay of transiently transfected cells. Fluorescent images were derived from cells either transfected with the same amount of pCMV-DV2Rep or replication-defective NS5 mut replicon. IFA images were taken at 100× magnification showing expression of DENV NS3 protein in transfected cells at 72 h post-transfection. Green cells indicate positive cells. The cells were nuclear stained with DAPI (blue color) (D) Western blot analysis of DENV NS3 protein. The expression level of NS3 protein was compared with the BHK21 cells transfected with the same amount of pCMV-DV2Rep or replication-defective NS5 mut replicon. GAPDH is also detected as loading control. (E) The dose–response curves derived from DNA-launched DENV replicons. The IC_{50} values of 2-C-methyladenosine (Carroll et al., 2003), mycophenolic acid (Diamond et al., 2002) and BP2109 (Yang et al., 2011) were measured in cells transiently expressed DENV replicons. BHK21 cells were transfected with a pCMV-DV2Rep plasmid and then treated with each compound at 24 h post-transfection. The mean values and SEMs from three independent experiments are plotted at 72 h post-transfection. The dose–response curve was plotted with Prism software using a sigmoidal curve fitted with a variable slope. (F) Compound susceptibility of the DNA-launched replicons. The susceptibility of 2′-C-methyladenosine, mycophenolic acid and BP2109 were plotted with Prism software using a sigmoidal curve fitted with a variable slope. The IC_{50}, which represents a 50% reduction in the *Renilla* luciferase activity assay, was calculated.



C

P10	Average IC_{50} (μM)	Average CC_{50} (μM)
2′-C-methyladenosine	4.58 ± 0.77	> 30
Mycophenolic acid	0.90 ± 0.11	> 15
BP2109	1.21 ± 0.30	> 25



E

2′-C-methyladenosine	P10	P30
IC_{50} (μM)	4.58 ± 0.77	5.21 ± 0.98
Rluc signal/10^4 cells	$8.01\times10^8 \pm 5.31\times10^6$	$4.71\times10^8 \pm 1.85\times10^7$

Fig. 6. No apparent difference in replication activity or compound susceptibility was observed among different passages of DENV replicon cells. (A) Stable expression of colonies derived from individual colonies. BHK21 cells were transfected with the pCMV-DV2Rep plasmid and then selected with 1 mg/ml G418 for 2–3 weeks. Six colonies were individually picked and their Rluc signal was monitored at the indicated passages. The solid line indicates the SEM of six colonies. (B) The dose–response curves of the 10th passage (P10) of Clone 5 DENV replicon cells. The IC_{50} values of 2′-C-methyladenosine (Carroll et al., 2003), mycophenolic acid (Diamond et al., 2002) and BP2109 (Yang et al., 2011) were measured at the 10th passage (P10) of Clone 5 DENV replicon cells. The values represent the mean values and SEMs from three independent experiments. (C) Compound susceptibility assay performed during the 10th passage of Clone 5 DENV replicon cells. The IC_{50} and CC_{50} values of 2′-C-methyladenosine, mycophenolic acid and BP2109 were measured at the 10th passage (P10) of Clone 5 DENV replicon cells. The values represent the mean values and SEMs from three independent experiments. (D) The dose–response curves of the DENV replicon cells. The 10th and 30th passages (P10 and P30) of Clone 5 DENV replicon cells were used to measure the IC_{50} values of 2′-C-methyladenosine. The 2′-C-methyladenosine susceptibility at different passages of the replicon cells was plotted with Prism software. The mean values and SEMs from three independent experiments are plotted. (E) 2′-C-methyladenosine susceptibility at different passages of Clone 5 DENV replicon cells. The IC_{50} values and Rluc-reporter signals (relative luminescence signal for 1×10^4 cells) of the mock control were also recorded. The values represent the mean values and SEMs from three independent experiments.

post-transfection in both BHK21 (~13-, 3-, and 8-fold increase, respectively) (Fig. 3A) and Huh7.5 cells (~13-, 2-, and 4-fold increase, respectively) (Fig. 3B). These results indicated that the truncation mutations within the capsid-coding region of the DENV replicon affect its RNA synthesis level. Altogether, these results indicate that the RNA sequences from nts 163–207 and 307–378 of the DENV genome are important for viral replication capacity.

3.2. Stable cells harboring the DENV replicon containing the entire mature capsid gene sequence displayed efficient viral replication

To further evaluate the role of the RNA sequence containing nts 307–378 of the DENV genome in viral RNA replication, we transfected nt207 or nt402 replicon RNAs into BHK21 cells and isolated individual stable clones harboring the DENV replicon derived from a single cell in the presence of 1 mg/ml G418 selection drug. As a

Table 2
Sequencing analyses of DENV NS1–NS5 gene from clones derived from DENV replicon cells (Clone 5 cells) at passage 30.

Amino acid of parental DENV genome	811	853	1272	1290	1345	1425	1598	1751	2099	2114	2125	2130	2181	2465	2995	3031	3319
	P	I	L	N	R	E	I	P	L	A	H	G	I	A	L	T	T
Clone																	
1				H						V	Y						
2	L		V			K											
3				H							Y	C	M	V	P		
4				H			T	S	P		Y		M			I	A
5		F		H	M						Y		M	V			

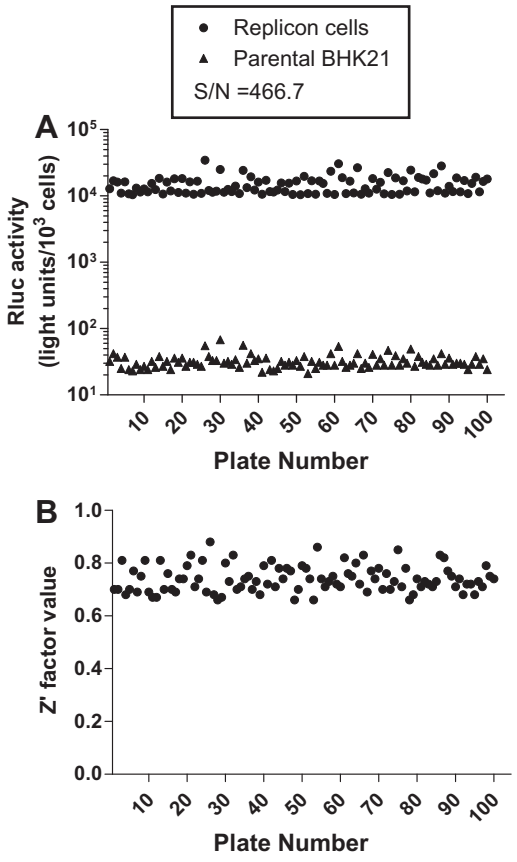


Fig. 7. Evaluation and validation of a high throughput screening system for the DENV replicon cells in 96-well plates. (A) The assay data from one hundred screens. Replicon cells and parental BHK21 cells were used in each screen as the signal and the background control (relative luminescence signal for 10^3 cells) respectively. The mean of the S/N (signal to noise ratio) was also measured and is shown above (mean signal over mean background). (B) The *Z′* factor of each plate was calculated and is plotted with a mean of 0.74 and a SD of 0.05.

control, cells transfected with the sp6-NS5 mut replicon did not form any cell colonies after G418 selection for two weeks. Fifteen colonies of the nt207 or the nt402 stable replicon cells were randomly selected, tested and compared side by side. As shown in Fig. 4, on average, the cells containing the nt402 replicon clones had a 2.3-fold higher luciferase activity than the cells containing the nt207 replicon clones ($p < 0.0001$). These results further validate that the sequence spanning nts 307–378 of the DENV genome may possibly increase the replication efficiency of the viral RNA in BHK21 cells.

3.3. Development and characterization of an efficient DNA-launched DENV replicon plasmid, pCMV-DV2Rep

Based on the knowledge gained from the RNA-launch replicons, we sought to establish an efficient and convenient transient replicon assay system for validation of compounds, by constructing a DNA-launched DENV replicon, pCMV-DV2Rep, containing the entire mature capsid gene sequence (Fig. 5A). This system allows convenient transfection of the DENV subgenome into cells in the form of plasmid DNA without *in vitro* transcription (Varnavski et al., 2000). The resulting plasmid contains a sub-genomic cDNA of the nt402 replicon in which the 5′-UTR of the DENV genome is located immediately downstream of the minimal cytomegalovirus (CMV) promoter and the 3′-UTR is located just upstream of the antigenomic sequences of the hepatitis delta virus ribozyme (HDVr) (Fig. 5A). The HDVr sequences ensure the generation of the correct DENV 3′ terminus. The simian virus 40 polyadenylation signal was inserted at the end of the DENV replicon to stabilize the replicon RNA in cells.

To characterize the replication efficacy of pCMV-DV2Rep in mammalian cells, luciferase assays were performed at the indicated time points by transfecting BHK21 cells with pCMV-DV2Rep. As a control, to ensure that amplification of the Rluc signal was mediated through RNA replication, a replication-defective replicon was designed by mutating the GDD motif of NS5 to GAA (NS5 mut). The NS5 mut replicon did not replicate (Fig. 5B). As shown in Fig. 5B, the levels of Rluc activity peaked at 72 h post-transfection, which reflects replication of the input DNA. The normalized Rluc signal at 72 h was used to evaluate replication efficiency. To determine the replicon expression of DNA-launched DENV replicon plasmid in cells, we transfected pCMV-DV2Rep and NS5 mut plasmids into BHK21 cells and found that the expression of DENV NS3 proteins within cells transfected with pCMV-DV2Rep plasmid (~3% cells with positive NS3 staining) but not with NS5 mut by immunofluorescence assay and Western blotting at 72 h after transfection (Fig 5C and D). These results indicate that the pCMV-DV2Rep replicon can replicate efficiently and express DENV and foreign proteins in mammalian cells.

To assess the biological relevance of the DNA-launched DENV replicon construct, we transiently expressed pCMV-DV2Rep in the presence of various known antiviral compounds, e.g., 2′-C-methyladenosine (Carroll et al., 2003), mycophenolic acid (Diamond et al., 2002) and BP2109 (Yang et al., 2011). As shown in Fig. 5E and F, we determined the IC_{50} (the inhibitory concentration that resulted in 50% of the control luciferase signal) values of 2′-C-methyladenosine (Carroll et al., 2003), mycophenolic acid (Diamond et al., 2002) and BP2109 (Yang et al., 2011) to be 1.47 ± 0.16, 0.16 ± 0.01, and 0.39 ± 0.05 μM, respectively, using a DNA-launched DENV replicon assay system. The IC_{50} value of BP2109 is consistent with the IC_{50} value of the same compound in RNA-launched DENV reporter replicon assays (0.17 ± 0.01 μM) (Yang et al., 2011). Moreover, the mycophenolic acid susceptibility of the DNA-launched replicon is also consistent with the IC_{50} values of the same compounds in whole viral infection assays that were performed in Hep3B cells using flow cytometry to detect the DENV envelope protein (0.31 μM) (Diamond et al., 2002). These results indicate that measurement of the Rluc signal produced by the DNA-launched DENV replicon is similar to the transient RNA-launched replicon replication assay and that the DENV infection assay can be used for anti-DENV compound validation. The

replication assay of the DNA-launched DENV reporter can also be used as an appropriate and efficient method to quickly and conveniently measure or compare the inhibitory activity of compounds.

3.4. Stable cells derived from the pCMV-DV2Rep construct steadily and efficiently replicate at 10 and 30 cell passages

In order to obtain stable dengue replicon cells that efficiently and steadily express for long-term period of time, we sought to determine if our DENV replicon, which harbors the entire mature capsid gene sequence, would persistently replicate in cells well. We transfected BHK21 cells with pCMV-DV2Rep plasmid and isolated stable cell colonies under G418 selection for 2 weeks. As a control, cells transfected with the NS5 mut replicon did not form any cell colonies after G418 selection for two weeks. Six clones were randomly selected and amplified to monitor and compare luciferase activity. As shown in the Fig. 6A, all DENV replicon cell clones demonstrated stable luciferase activity for over 30 passages. In addition to the expression of reporter luciferase, the expression of DENV NS3 proteins within Clone 5 stable cells at passage 30 was examined. As shown in Supplementary Fig.1A and B, the expression of viral proteins, e.g. DENV NS3 protein, was detected in both of most of Clone 5 stable cells at passage 10 and 30 by immunofluorescence assay and Western blotting using antibody against DENV NS3 protein. These results strongly suggested that the stably transfected DENV replicon cells harboring the entire mature capsid gene sequence in the replicon context have both high replication capacity (>10^7 light units/10^4 cells) and stability, which makes them suitable for HTSs and for evaluation of the efficacy of inhibitors. To examine if there are mutations occurred during the passage of stable replicon cells, we performed sequencing analyses to determine the DENV replicon RNAs derived from Clone 5 stable cells at passage 30. Only two major mutations, N1290H in NS2A and H2125Y in NS4A, were found in 4 out of 5 clones (Table 2). Two minor mutations were found I2181M (3 out of 5 clones) in NS4A and A2465V (2 out of 5 clones) in NS4B.

To measure the compound susceptibility of stable DENV replicon cells, we quantified the inhibitory activity and cytotoxicity of known antiviral compounds. We measured the IC_{50} and CC_{50} (the concentration that resulted in 50% of the control cell number) values using the DENV replicon cells with the highest reporter signal, Clone 5 (>10^8 light units/10^4 cells). As shown in Fig. 6B and C, 2′-C-methyladenosine (Carroll et al., 2003), mycophenolic acid (Diamond et al., 2002) and BP2109 (Yang et al., 2011) showed replication inhibitory effects with IC_{50} values of 4.58, 0.9, and 1.21 μM, respectively.

To compare the compound susceptibility at different passages of Clone 5 DENV replicon cells, the dose–response curves and IC_{50} values for 2′-C-methyladenosine were plotted and measured during the 10th and 30th passages of the replicon cells. As shown in Fig. 6D and E, the Rluc signals and compound susceptibilities were consistent between different passages of DENV replicon cells. These results provided evidence that our replicon design could stably persist and express in BHK21 cells with a high Rluc signal, which makes them suitable for HTSs of anti-DENV compounds and for evaluation of the efficacy of inhibitors.

3.5. Evaluation of stable DENV replicon cells for high-throughput screening of DENV inhibitors

Once we successfully established a BHK21 cell line that stably expressed the DENV reporter replicon with a high luciferase signal, we intended to utilize the DENV reporter replicon system in a high-throughput screen (HTS) of DENV inhibitors. The pilot HTS is set up in a 96-well format and had a consistent and robust signal. To evaluate the assay performance, one hundred 96-well plates containing the replicon cells and the parental BHK21 cells were run, and tested with 8000 compounds with 15 μM BP2109 as a reference inhibitor. As shown in Fig. 7A, the 1000 cells containing the DENV replicon cells had an average Rluc signal of 14,971 with a SD of 4848, whereas the parental BHK cells had a background signal of 32 with a SD of 7.79. The assay system had a signal to background ratio of 466.7. The Z' factor was calculated and plotted for each plate that was tested and had a mean of 0.74 and a SD of 0.05 (Fig. 7B). Therefore, our screen format has adequate sensitivity, reproducibility, and accuracy to discriminate among a large number of compounds.

4. Discussion

In the present study, we developed an efficient, long-lasting DENV replicon system containing the full-length capsid gene sequence. Two regulatory RNA elements that are required for replication were discovered and mapped in the N- (nts 163–207) and C-terminal (nts 307–378) portion of the DENV capsid gene. Inclusion of the full-length capsid gene enabled us to create, an efficient DNA-launched DENV replicon, pCMV-DV2Rep, which we used to conveniently evaluate the potency of DENV inhibitors in a transient replicon assay. Stable cells expressing the DENV reporter replicon were established by transfecting cells with pCMV-DV2Rep and selecting for the drug resistance marker contained within the replicon. These stable cell lines steadily expressed high levels of DENV replication for over 30 passages without an apparent decrease in the reporter signal. The stably expressing DENV replicon cell line was shown to be suitable for high throughput screening of DENV inhibitors. Our findings suggested that the full-length capsid sequence is required for transiently or stably establishing an efficient DENV replicon. These results provide insight into the molecular mechanism of DENV replication and should facilitate the development of both anti-DENV drugs, and DENV vaccines.

Cis-acting elements that are involved in DENV replication have been shown to be essential for efficient replication, and most of the previously identified elements are located in the 5′ UTR and the 3′ UTR of the DENV genome (Paranjape and Harris, 2010). Our attempts to elucidate the role of the capsid sequence during viral replication and to create an efficient DENV replicon revealed that a deletion in the capsid sequence affected viral replication in two mammalian cell types (BHK21 and Huh7.5) with differential replication rates (Fig. 2). Additionally, the results indicated that the RNA sequence containing nts 163–207 is critical for modulating viral replication and the replicon containing nts 307–378 showed the greatest replication efficacy (Figs. 2–4). This observation is consistent with our previous report, which indicates that silent mutations introduced into nts 186–192 of DENV replicons and infectious cDNA significantly reduced both viral yields and the replication efficiencies observed in the transient replicon assay in BHK21 cells (Pu et al., 2011). The reduction of replicon activity through the introduction of silent mutations may result from the sequence and structure of nts 163–207 and nts 307–378 and their possible effects on the base pairing with the 3′ end of the DENV UTR or/and RNA structural formation of the 5′ *cis*-elements and the 3′ *cis*-elements. We suspected that truncations or silent mutations introduced into either the central or C-terminus region of the DENV capsid-coding region may interfere with viral replication by disrupting RNA secondary structures, RNA-RNA or RNA–protein interactions that are essential for viral replication. This speculation is supported by several previous reports. For example, the complementarity of the cyclization sequences (CS) that is present in the capsid-coding region (5′ CS, nts 135–144) and the 3′ UTR (3′ CS, nts 10618 to 10628) is required for RNA–RNA complex formation (Alvarez et al., 2005b) and is necessary for efficient RNA synthesis

by the DENV virus RNA-dependent RNA polymerase (Alvarez et al., 2005a; You et al., 2001; You and Padmanabhan, 1999). Another two RNA elements, the capsid hairpin (cHP, nts 114–134) and the 5′ downstream AUG region (5′ DAR, nts 105–110), which are located at the N terminus of the capsid-coding region, serve as *cis*-acting elements that are essential for virus replication (Clyde et al., 2008; Clyde and Harris, 2006; Friebe and Harris, 2010; Friebe et al., 2011). It was proposed that the cHP may stabilize the overall 5′-3′ panhandle structure or participate in the recruitment of proteins involved in the replicase machinery (Clyde et al., 2008) and that the 5′ DAR may be involved in viral genome circularization (Friebe and Harris, 2010; Friebe et al., 2011). Recently, a report suggested that the RNA sequence spanning nts 170–200 in the DENV genome, rather than the protein, affects viral RNA replication by modulating the topology of the viral 5′ end (Friebe et al., 2012; Groat-Carmona et al., 2012), which is also consistent with our findings. Another interesting observation was that the replication capacity of nt306 replicon is less active than nt207, indicating that maybe there is a negative regulatory element in the sequence between nt 208–306 or the RNA conformation of cis-acting elements within nt306 replicon is disturbed. Altogether, we discovered two genomic regions (nts 163–207 and nts 307–378) that are located within the capsid gene and may act as *cis*-acting regulatory elements to promote RNA structural stabilization, RNA–RNA interactions, or genome circularization. Further investigation is needed to determine the detailed molecular mechanisms by which nts 163–207 and nts 307–378 serve as regulatory RNA elements.

One intriguing observation in this study is that the replication capacity of the various DENV replicon constructs at 72 h post transfection is higher in human Huh7.5 cells than in Chinese hamster BHK21 cells (Fig. 2). Four DENV replicon variants, nt162, nt207, nt306, and nt402, displayed a higher replication rate in Huh7.5 cells than in BHK21 cells with a 2-, 2-, 5- and 2-fold increase, respectively, at 72 h post-transfection (Fig. 2). It is not likely that the differences in the replication capacity between the two cell lines is due to differences in transfection efficiency of the DENV replicon RNA into cells because the replicon reporter activity of the various replicon constructs at 4 h post-transfection is similar. The cell type dependent phenomenon is in agreement with the previous report that indicated that the deletions within the 3′ *cis*-acting elements of JEV replicons affect viral replication depending on the cell type (Yun et al., 2009). Furthermore, it was proposed that additional cellular/viral factors or different strategies for JEV RNA replication are required in different host species (Yun et al., 2009). Thus, the differences in the replication capacity of DENV replicons in cells from different species are possibly due to the heterogeneity and the availability of replication machinery in the hamster and human cells.

The DNA-launched flavivirus replicon has been shown to have many advantages over RNA-launched flavivirus replicons, e.g., it requires less effort to prepare and handle (Ansarah-Sobrinho et al., 2008; Cao et al., 2011; Huang et al., 2012; Leardkamolkarn et al., 2012; Lee et al., 2010; Pang et al., 2001; Varnavski et al., 2000). Therefore, the DNA-launched DENV replicon, which is derived from the DENV 16681 strain (Fig. 5) that we developed, is ideally suited to evaluate the potency of known DENV inhibitors using either the transient replicon assay or cell line stably expressing the replicon. The IC_{50} values that were obtained for known DENV inhibitors (Fig. 5E and F) using a DNA-launched replicon assay are comparable to those obtained using an RNA-launched replicon assay (Ng et al., 2007). There is one concern that the replicon viral RNAs within stable DENV replicon cells derived from DNA-launched DENV replicon are resulting from the integration of DENV replicon plasmid into the chromosome DNAs of cells. Our sequencing analyses of Clone 5 cells at passage 30 revealed that few mutations within DENV replicon of Clone 5 cells (Table 2), indicating the sequences of replicon RNAs within Clone 5 cells are different from those of parental DENV replicon plasmid. Thus, our Clone 5 cells are not likely derived from the integration of replicon plasmid into chromosome DNAs. There is one interesting phenomenon that we easily obtained stable cell colonies harboring the DENV replicon by transfecting the DNA-launched DENV replicon plasmid, pCMV-DV2Rep, into cells. In contrast, greater effort is required to produce more DENV replicon cell colonies when transfecting the RNA-launched replicon viral RNAs into cells (unpublished data). We speculate that the ease of obtaining cell colonies derived from the DNA-launched DENV replicon is because of the lower toxicity that is caused by transient transfection with the DNA-launched replicon and not because of differences in transfection efficiency. This speculation is supported by the fact that fewer stable cell colonies were obtained when transfecting the selected adaptive replicon AdDENVRep, harboring mutations in the NS1–NS4B region of the parental DENVRep genome, RNAs than the parental DENVRep replicon RNAs. AdDENVRep has an approximately 2-fold higher replication capacity in the transient replicon assay than the parental DENVRep at 72 h post transfection (unpublished data), which implies that the higher replication rate results in less colony formation and implies that the higher replication rate may cause some toxicity to the cells. Further studies are needed to address the issue.

A number of viral replicon-based assays have been developed to screen for anti-DENV compounds (Hsu et al., 2012; Masse et al., 2010; Noble et al., 2010; Qing et al., 2010; Shum et al., 2010; Xie et al., 2011). The DENV replicon containing the full-length capsid sequence in this study showed higher replication capacity in BHK21 and Huh7.5 cells compared to DENV replicons containing partial capsid sequences (Fig. 2A and B). The stable BHK21 cells expressing the DENV reporter replicon harboring the full-length capsid sequence displayed steady and high Rluc signal, even after 30 passages (Fig. 6A) and 50 passages (data not shown). These stable cells were used for the pilot HTS of small molecules that affect DENV replication with a Z' value of 0.74 (Fig. 7A and B), which is an excellent HTS assay performance (Zhang et al., 1999). HTS was therefore conducted using stable DENV replicon cells (Clone 5 cells) for DENV inhibitors from a total of 60,000 small-molecule compound library. Around 200 hit compounds with low cytotoxicity were screened to reduce 50% of DENV replicon activity at the concentration of 5 μM (data not shown). Further studies are underway to investigate the roles of those hit compounds in inhibiting DENV replication.

The steady expression (at passage 30) of our DENV replicon cells stands in contrast to a previously reported DENV replicon, which was derived from strain NGC; the replicon reporter signal of that DENV replicon cell line was only stable for up to 13 passages in BHK21 cells (Ng et al., 2007). The HTS assay for DENV inhibitors using A549 cells harboring the DENV NGC replicon had a Z' value of only 0.4 (Xie et al., 2011). The molecular mechanism that accounts for difference in the duration of the reporter signal between NGC- and 16681-derived stable DENV replicon cells is not clear. The DENV NGC replicon construct reported in the previous report only contained the C22 portion of the capsid sequence (Ng et al., 2007), so therefore the addition of two regulatory sequences (nts 163–207 and nts 307–378 of the DENV capsid gene) within our DENV replicon construct may play a key role in establishing a stable replicon-bearing cell line. Furthermore, our construct is derived from DENV strain 16681, so some degree of variation in the amino acid sequence of the DENV genomes between strains NGC and 16681, may also contribute to the differences observed. Thus, we speculate that the steady and high replicon activity observed in our stable replicon cells may result from the different replication capacities and/or the different degrees of toxicity to the cells derived from replicon design.

In summary, a DENV reporter replicon containing the entire mature capsid gene sequence (nts 97–402 of the DENV genome) replicates more efficiently in a transient replicon assay than DENV reporter replicons that contain deletions in the capsid gene. Furthermore, stable BHK21 cells harboring a DENV replicon with a full-length capsid sequence maintain a high reporter signal and the replication stability to allow more efficient establishment of noncytopathic, stably persistent replication of the DENV replicon in BHK21 cells. Our replicon system provides new insights into the processes governing DENV replication and the design of DENV replicons for drug screening and validation. An additional HTS assay is planned to identify more potent small molecule inhibitors of the DENV replicon that may potentially be used for anti-DENV therapy.

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