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Short Communication

Construction of a dengue virus type 4 reporter replicon and analysis of temperature-sensitive mutations in non-structural proteins 3 and 5

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Replicon systems have been useful to study mechanisms of translation and replication of flavivirus RNAs. In this study, we constructed a dengue virus 4 replicon encoding a *Renilla* luciferase (R_{luc}) reporter, and six single-residue substitution mutants were generated: L128F and S158P in the non-structural protein (NS) 3 protease domain gene, and N96I, N390A, K437R and M805I in the NS5 gene. The effects of these substitutions on viral RNA translation and/or replication were examined by measuring R_{luc} activities in wild-type and mutant replicon RNA-transfected Vero cells incubated at 35, 37 and 39 °C. Our results show that none of the mutations affected translation of replicon RNAs; however, L128F and S158P of NS3 at 39 °C, and N96I of NS5 at 37 and 39 °C, presented temperature-sensitive (*ts*) phenotypes for replication. Furthermore, using *in vitro* methyltransferase assays, we identified that the N96I mutation in NS5 exhibited a *ts* phenotype for *N*7-methylation, but not for 2′-*O*-methylation.

The four serotypes of dengue virus (DENV1–4), mosquito-borne members of the family *Flaviviridae*, cause widespread human diseases ranging from dengue fever to the more severe forms, dengue haemorrhagic fever and dengue shock syndrome (Gubler, 2006; Halstead, 2008). DENV causes an estimated 50–100 million cases of dengue fever, of which 1 % result in severe forms. Currently, there is no vaccine or antiviral drug to treat DENV infections (Gubler, 2006; Hatch *et al.*, 2008; Kyle & Harris, 2008).

DENV has an ssRNA genome of positive polarity, approximately 11 kilobases in length, with a 5′ cap but no poly(A) tail at the 3′ end (reviewed by Lindenbach & Rice, 2003). The single long ORF of the viral RNA encodes a polyprotein that is processed co- and post-translationally by both cellular signal peptidase and viral serine protease in the endoplasmic reticulum (ER) membrane into three structural proteins, capsid (C), precursor membrane (prM) and envelope (E), and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (reviewed by Beasley, 2005; Harris *et al.*, 2006).

Flavivirus NS proteins play an essential role in viral RNA translation and replication. Some NS proteins have multiple functions in the virus life cycle (Miller *et al.*, 2010; Padmanabhan *et al.*, 2006). The N-terminal 180 residues of NS3, in conjunction with NS2B, function as a viral protease required for polyprotein processing (reviewed by Padmanabhan & Strongin, 2010; Sampath & Padmanabhan, 2009). The NS3 carboxy-terminal region has conserved motifs found in the 'D-E-X-H' family of RNA helicases (reviewed by Lescar *et al.*, 2008). The amino-terminal region of NS5 has a 5′ RNA methyltransferase (MTase) activity, which catalyses sequentially the transfer of a methyl group from *S*-adenosylmethionine to the N7 of the 5′ guanosine cap (Ray *et al.*, 2006; reviewed by Davidson, 2009; Dong *et al.*, 2008) and to the 2′-OH moiety of adenosine at the 5′ end of the RNA genome (Egloff *et al.*, 2002). NS5 also has RNA-dependent RNA polymerase (RdRP) activity (Tan *et al.*, 1996), capable of *de novo* synthesis of the minus strand *in vitro* (Ackermann & Padmanabhan, 2001), and is required for virus replication in cultured cells (Khromykh *et al.*, 1998, 1999; reviewed by Malet *et al.*, 2008; Miller *et al.*, 2010).

Replicons encoding a reporter gene are useful tools for studying the translation and replication of flaviviruses, including Kunjin virus (Khromykh & Westaway, 1997), West Nile virus (WNV) (Shi *et al.*, 2002), yellow fever virus (Corver *et al.*, 2003; Jones *et al.*, 2005a), DENV2 (Alvarez *et al.*, 2005; Jones *et al.*, 2005b; M. I. M. Manzano, E. D. Reichert, S. L. Alcaraz-Estrada, S. Polo, B. Falgout, W. Kasprzak, B. A. Shapiro & R. Padmanabhan, unpublished results) and

A supplementary figure, supplementary methods and two supplementary tables are available with the online version of this paper.

DENV1 (Puig-Basagoiti *et al.*, 2006). However, to our knowledge, the construction of a reporter replicon for DENV4 has not yet been reported.

We sought to construct a DENV4 replicon with *Renilla* luciferase (R_{luc}) reporter to study mechanisms of virus replication. A DENV4 infectious clone was placed into an *Escherichia coli*–yeast shuttle vector to yield the pRS424-DENV4IC clone by using a previously described method (Polo *et al.*, 1997). The pRS424-DENV4IC clone was used for the construction of a DENV4 replicon (DENV4Rep) as shown in Fig. 1(a) [see Supplementary Fig. S1 (available in JGV Online) for details].

The DENV4 replicon contains the 5′UTR and the first 25 codons of the capsid (C) gene, fused to the ORF of the R_{luc} reporter with its own initiation and termination codons. This region of the C gene contains the 5′ conserved sequence (CS), which interacts with the 3′CS1 in the 3′UTR (Hahn *et al.*, 1987). Expression of the R_{luc} reporter

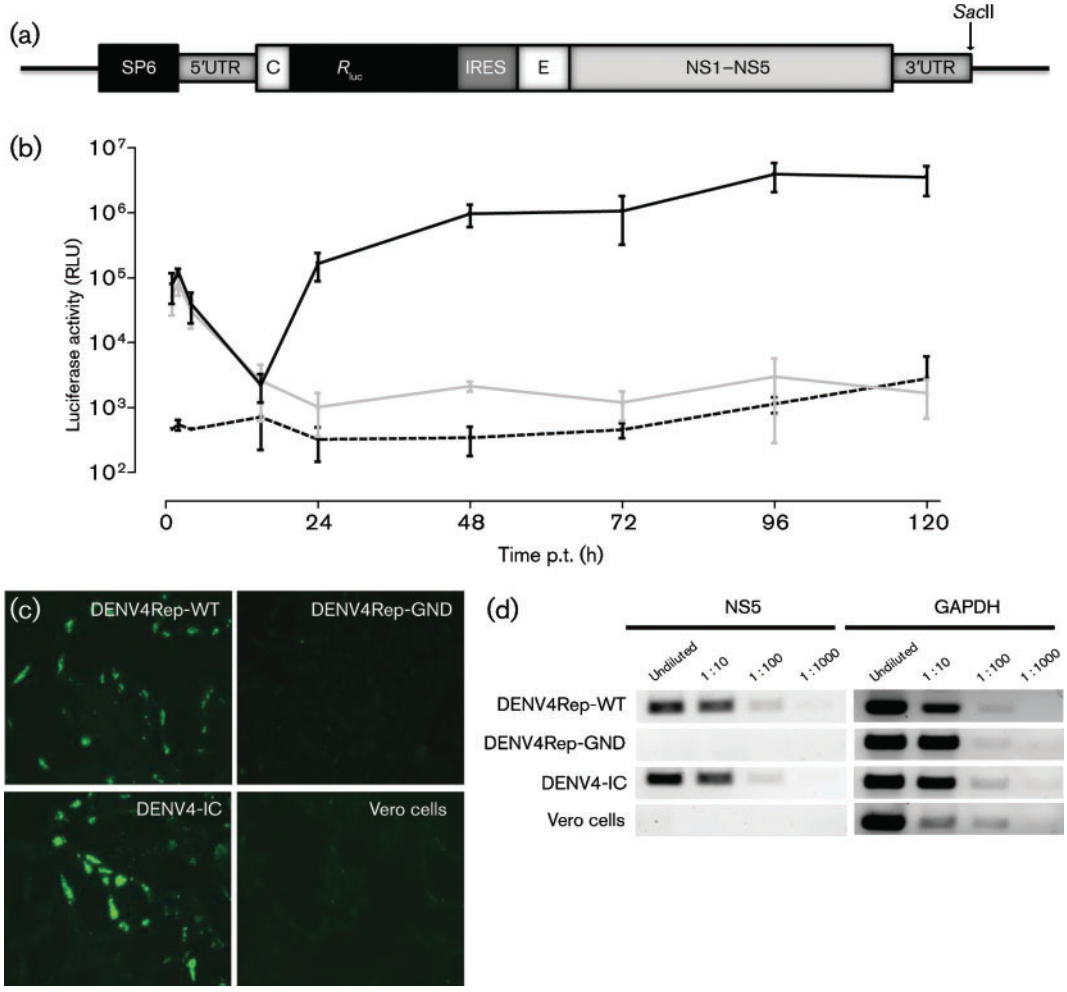

Fig. 1. Characterization of DENV4Rep-WT and -RepGND mutant replicons. (a) Schematic representation of DENV4rep-WT. Details of the replicon construction are described in Supplementary Fig. S1. (b) Vero cells were transfected with RNAs from DENV4Rep-WT (black line) and -GND mutant (grey line) replicons. At different times after transfection, *Renilla* luciferase (R_{luc}) activity was measured in extracts of transfected cells, or cells incubated without any RNA transfection (dotted line), as relative light units (RLU) with a Centro LB 960 luminometer (Berthold Technologies). (c) Immunofluorescence. RNA-transfected Vero cells were grown on glass coverslips. Detection of viral NS5 protein in DENV4Rep-WT- and DENVRep-GND-transfected cells at 96 h post-transfection (p.t.) by immunofluorescence using rabbit polyclonal anti-DENV2 NS5 antibodies (M. Ackermann & R. Padmanabhan, unpublished results) is shown. Details are given in Supplementary Methods (available in JGV Online). (d) Estimation of viral RNAs by semiquantative RT-PCR. Total RNA was extracted from Vero cells at 96 h p.t. Tenfold dilutions of RNA from 250 to 0.25 ng were subjected to reverse transcription (RT) at 42 °C. An aliquot of the RT reaction mixture (1 μl) was used for PCR, using primers from the NS5 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control) genes. Details are given in Supplementary Methods. Untransfected Vero cells were used as a negative control. Each transfection was performed in triplicate and each experiment was repeated at least three times.

is under cap-dependent translational control. The 3′ end of the R_{luc} gene is fused to the encephalomyocarditis virus internal ribosome entry site (IRES) to direct cap-independent translation of the long ORF encoding the viral NS proteins (Fig. 1a). The region downstream from the IRES contains the C-terminal 37 codons of the viral envelope (E) gene that is required for proper translocation of NS1 and other NS proteins into the ER. In addition, we constructed a DENV4 replicon containing the GDD→GND mutation in the RdRP gene, which is known to abolish viral RNA replication (Khromykh *et al.*, 1998; Ribas & Wickner, 1992).

The time course of R_{luc} expression was studied in Vero cells. RNAs from DENV4rep wild type (WT) and mutants were obtained by *in vitro* transcription (for details, see Supplementary Methods, available in JGV Online). Vero cells (approx. 10^6) were transfected with the RNAs (approx. 3 μg) by electroporation. Cells were plated into six wells of a 48-well plate for R_{luc} assays (100 μl per well) and the rest into a 12-well plate containing 1 ml medium for RT-PCR.

Transient expression of DENV4rep and DENV4rep-GND in transfected cells was monitored by R_{luc} activity assays with lysates of transfected cells collected at different times post-transfection (p.t.) (Fig. 1b). The first peak of R_{luc} activity occurred at 2 h p.t. due to translation, and then declined. At 24 h p.t., the R_{luc} signal from WT DENV4rep began to increase until it reached a maximum at 96 h p.t., due to translation of the replicated viral RNA. However, for the DENV4rep-GND mutant replicon, the R_{luc} activity reached a peak at 2 h p.t. due to translation, but decreased steadily to background levels due to a deficiency in replication (Fig. 1b).

Replication of the WT DENV4rep was confirmed by immunofluorescence assay of NS1 in the WT replicon-transfected cells at 96 h p.t., but was negative in DENV4rep-GND- or non-transfected cells (Fig. 1c). Finally, replication of DENV4rep was confirmed by RT-PCR using primers specific for the DENV4 NS5 gene at 96 h p.t. Again, a PCR-amplified product of the expected size was obtained with DENV4rep but not with the DENV4rep-GND replicon RNA-transfected cells (Fig. 1d). Taken together, these results indicated that the DENV4rep RNA is functional and is suitable to study virus translation and replication by measuring luciferase activity at 2 and 96 h p.t., respectively.

Temperature-sensitive (*ts*) mutants of a gene provide a powerful tool for studying protein function because they show a marked change in the level or activity of the gene product when expressed at a non-permissive temperature. At permissive temperatures, the phenotype of the mutant is close to that of the WT. For YFV, a *ts* mutant of NS1 shown to be defective in accumulation of viral RNA at a

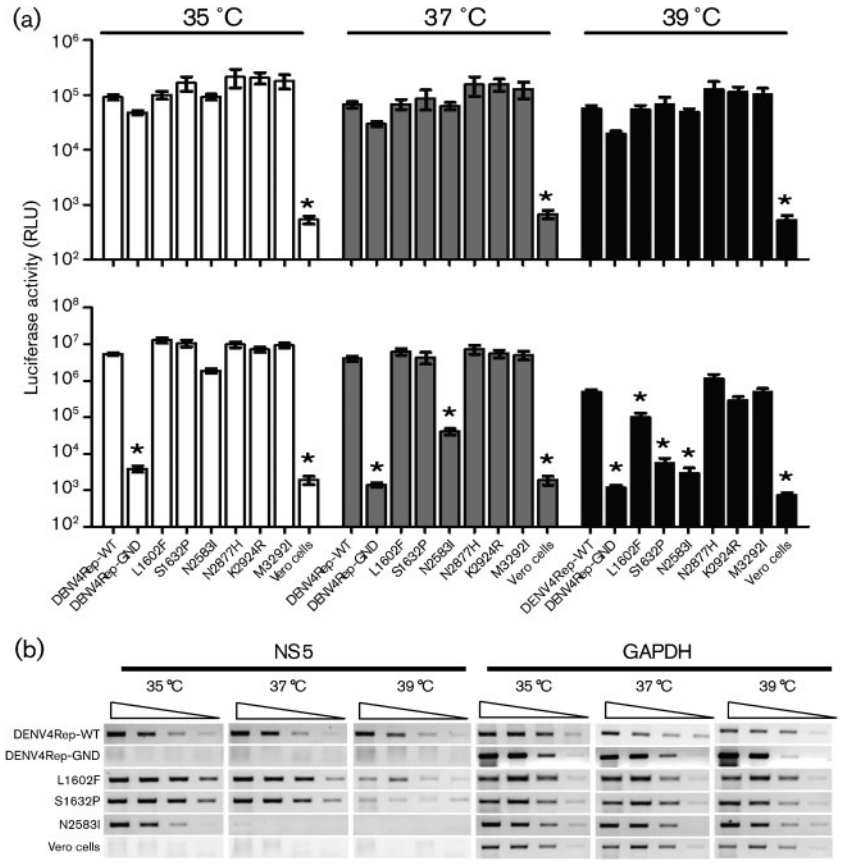

Fig. 2. Translation and replication efficiency of replicons at different temperatures. (a) Vero cells were transfected with a replicon RNA and incubated at 35, 37 or 39 °C for 2 h to determine translation efficiency (upper panel) or for 96 h to evaluate replication (lower panel). The designation of each mutant replicon by the residue number from the N terminus of mature NS3 or NS5, as well as in the polyprotein, is shown in Supplementary Table S2 (available in JGV Online). Luciferase activity was measured in extracts of transfected cells; the values obtained with the WT and mutant replicons were compared with those obtained with the GND replicon. R_{luc} activities (expressed as RLU) of each group were compared with that of the WT by using a Mann–Whitney test (α=0.01) (Minitab 15 statistical software); significant differences (P<0.001) are indicated by asterisks. (b) Estimation of viral RNA by RT-PCR at 96 h p.t. using primers for NS5. RT-PCR amplification of the GAPDH gene was used as loading control. Non-transfected Vero cells were used as negative controls.

non-permissive temperature revealed a role of this protein in viral RNA replication (Muylaert *et al.*, 1997). One commonly used method to generate *ts* mutants is by chemical mutagenesis, followed by screening of a large number of progeny for identification of a particular phenotype resulting from either loss or gain of function. Blaney and colleagues isolated *ts* mutants by treatment of DENV4-infected cells with the mutagen 5-fluorouracil and characterized mutants that exhibited *ts* phenotypes in both Vero and HuH-7 cells (Blaney *et al.*, 2001; Hanley *et al.*, 2002). Several attenuated *ts* mutations affecting virus growth at non-permissive temperatures were identified. However, as some of the *ts* mutants had substitutions of multiple residues in NS genes, the mutation causing a *ts* phenotype could not be ascribed to a single residue. Moreover, it was not known at what step of the virus life cycle the *ts* phenotype was manifested.

We focused our efforts to study *ts* mutations in NS3 and NS5, as these proteins play important roles in virus replication (Gamarnik, 2010; Miller *et al.*, 2010; Padmanabhan *et al.*, 2006). To identify mutations of single residues contributing to the *ts* phenotypes, six single-residue substitution mutants of DENV4rep were constructed (see Supplementary Table S2, available in JGV Online). WT and mutant replicon RNAs were synthesized by *in vitro* transcription of linearized replicon plasmids catalysed by phage SP6 RNA polymerase. Equal amounts of replicon RNAs were transfected by electroporation into Vero cells and the transfected cells were incubated at 35, 37 and 39 °C. Cells were lysed at 2 and 96 h p.t. and luciferase activities were measured. At 2 h p.t., the luciferase activities derived from each mutant at different temperatures did not differ to an appreciable extent from those obtained from the WT DENV4rep-transfected cells (Fig. 2a), suggesting that none of the mutations exhibited a *ts* phenotype for viral RNA translation. In contrast, the *ts* mutations S158P in the NS3 protease (NS3pro) domain at 39 °C and N96I in the NS5 MTase domain at 37 and 39 °C showed significantly reduced luciferase activity ($P<0.001$, Mann–Whitney test) compared with the DENVrep-WT replicon at 96 h p.t. (Fig. 2a).

To confirm the results from the R_{luc} activity assays done at 96 h p.t., the levels of replicated viral RNA from the WT and mutant replicons were determined by semiquantitative RT-PCR. The results were in good agreement with the R_{luc} signals, as seen from the significant reduction of RNA levels at 37 and 39 °C for the NS5 N96I mutant, as well as for the two NS3pro mutants, L128F and S158P, at 39 °C (Fig. 2b). Thus, the results taken together indicate that the single-residue substitution mutants in the NS3pro domain and the NS5 MTase domain exhibited *ts* phenotypes for RNA synthesis, with no appreciable effect on viral translation.

The NS5 MTase domain catalyses transfer of a -CH_3 group from *S*-adenosylmethionine to the N7 of the guanosine cap (N7 MTase) and to the 2′-OH of the first 5′ nucleotide of RNA (2′-O MTase). The 2′-O MTase activity of DENV2 NS5 was first demonstrated using capped and uncapped

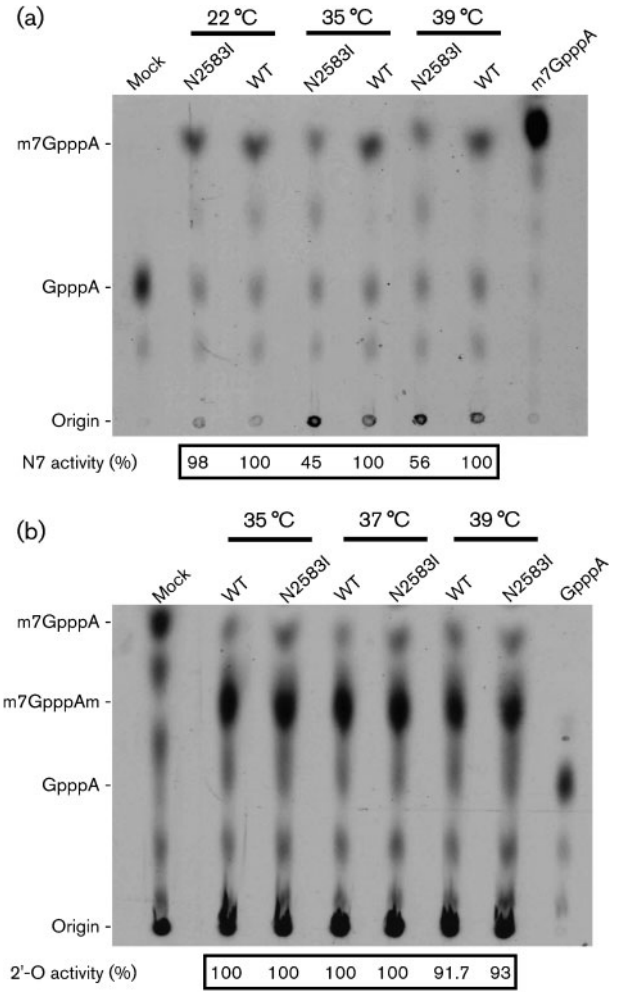

Fig. 3. N7 and 2′-O MTase activity assays. N7 and 2′-O MTase assays were done as described previously (Zhou *et al.*, 2007) (see also Supplementary Methods). The reactions were incubated at 35, 37 and 39 °C for 2 h and stopped by heating at 95 °C for 10 min. The designation of each mutant replicon is as described in Fig. 2 and Supplementary Table S2. (a) The reaction mixtures were treated with 1 U nuclease P1 overnight, and analysed on PEI-cellulose TLC plates using 0.45 M ammonium sulfate. Plates were dried and radioactivity was detected by autoradiography. (b) 2′-O MTase activity was measured by conversion of m^7GpppA-RNA to m^7GpppAm-RNA at pH 10 at the indicated temperatures. The relative activity of *N*7- and 2′-*O*-methylation is shown at the bottom of each panel, with the WT activity set at 100 %. The positions of the origins and migrations of the GpppA, m^7GpppA and m^7GpppAm molecules are indicated on the left side of the autoradiograms. Results representative of at least three independent experiments are presented.

short RNAs (GpppACCCCC and $^{7\text{-Me}}$GpppACCCCC) as substrates (Egloff *et al.*, 2002). Subsequently, Ray *et al.* (2006), using WNV 5′-terminal RNA as substrate for a -CH_3 acceptor, revealed that specific nucleotide sequence and 5′ stem–loop structure of the RNA are required for *N*7-methylation and that both *N*7- and 2′-O-Me additions

are sequential reactions catalysed by the NS5 MTase (Dong *et al.*, 2007; Ray *et al.*, 2006). *N*7-methylation is essential for WNV replication, whereas the defect in 2′-*O*-methylation attenuates the virus (Zhou *et al.*, 2007). We sought to investigate whether the *ts* N96I mutation of NS5 affects the activity of N7 cap- and/or 2′-*O*-methylation. To this end, the WT and N96I mutant NS5 MTase domains, each with a His tag at the N terminus, were expressed in a soluble form in *E. coli* and the recombinant proteins were purified (data not shown). The N7 and 2′-O MTase activities of these recombinant proteins were assayed at different temperatures as described previously (Zhou *et al.*, 2007). N7 cap-methylation, usually evaluated at 22 °C, was reduced significantly ($P<0.001$, Mann–Whitney test) in the *ts* mutant NS5 MTase domain at 37 and 39 °C, suggesting that the N96I mutation causes a *ts* phenotype for NS5 function in N7 MTase activity (Fig. 3a), although the 2′-O MTase was not affected significantly ($P=0.1$, Mann–Whitney test; Fig. 3b). Further studies are required to understand the molecular basis of the N96I mutation in conferring a *ts* phenotype on DENV4 NS5 function.

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