Arch Virol (2013) 158:583–599
DOI 10.1007/s00705-012-1525-2

ORIGINAL ARTICLE

Detection and sequencing of defective viral genomes in C6/36 cells persistently infected with dengue virus 2

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Received: 4 December 2011 / Accepted: 24 September 2012 / Published online: 6 November 2012

Abstract Dengue virus is the most important arbovirus that affects humans, and it can establish persistent infections, especially in insect-derived cell cultures. Defective viral genomes have been implicated in the establishment and maintenance of persistent infections with several flaviviruses; however, there exists almost no information concerning defective dengue virus genomes. Here, we report the detection of defective dengue 2 virus genomes in persistently infected mosquito C6/36 cells. The defective viral genomes were detected at a low ratio compared with the wild-type genome. Deletions of approximately 147 residues (222-368) were found in the E protein, and these mainly affected domain III (73 %) of the protein; deletions of approximately 153 residues (4-156) and 228 residues (597-825) were found in the methyltransferase and polymerase domains, respectively, of the NS5 protein. The truncated versions of NS5 could be detected by western blot only in the protein extracts derived from persistently infected cells.

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Introduction

Dengue virus (DENV) is an enveloped, single-stranded, positive RNA virus and a member of the family *Flaviviridae*. It is transmitted to humans by mosquitoes and causes a serious febrile illness known as dengue fever (DF). Complications associated with DENV infection include dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1]. Although DF affects more than 100 million people worldwide, there are no antiviral agents available [1, 2], and a vaccine is still being tested clinically [3]. Although DENV infection in humans is acute and self-limiting, in mosquitoes it is life-long or persistent [4, 5].

Defective interfering (DI) particles have been described for a wide variety of viruses. They are antigenically identical to the wild-type virus but can inhibit growth of the wild-type virus by competing for limited and essential replication factors. DI particles are also responsible for the establishment and maintenance of persistent viral infections. The genomes of DI particles are characterized by rearrangements and deletions caused by the aberrant activity of the viral RNA-dependent RNA polymerase (RdRp); however, sequences essential for replication and encapsidation are usually preserved. These defective genomes contain all the major elements necessary for replication and assembly, but they are not infectious themselves and always require the participation of the parental virus to act as a "helper virus" [6, 7]. Recently, these defective genomes have been detected circulating in human and mosquito populations, and they have been implicated as cofactors in reducing the prevalence of DENV [8] as well as the severity of the disease [9].

Long-term viral infections in cell lines provide an *in vitro* model in which to study chronic disease and to

investigate the process by which persistent infections are established and maintained [10]. A persistent flavivirus infection can more easily be established in cell of mosquito origin. Defective viral genomes have been detected in mosquito cells persistently infected with Saint Louis encephalitis virus (SLE) [11] and C6/36 cells [12] infected with Japanese encephalitis virus (JEV) [13]. When C6/36 cells are persistently infected with all four DENV serotypes, the presence of thermosensitive mutants with attenuated neurovirulence in mice has been detected [14]. Similar results have been observed in another study performed with DENV 2 [15]. Additionally, a variant of DENV 1 transmitted between humans and mosquitoes for at least 18 months was described. This mutant exhibited a defective genome that contained a stop codon at residue E248 (nucleotide 742), resulting from a C-to-U transition [8]. Recently, short segments of the DENV genome containing only the regulatory elements at 3' and 5' ends were detected in sera from acutely infected patients [9]. Aside from the latter studies, no additional information is available on defective DENV genomes, especially those in persistently infected cells.

In this paper, we report for the first time the presence of defective viral genomes in C6/36 cells persistently infected with DENV. The relevance of this finding is discussed.

Materials and methods

Cells and viruses

C6/36 cells cloned by Igarashi [12] and adapted to grow at 35 °C by Kuno and Oliver [16] were cultured in 75-cm^2 flasks (Corning) in MEM (Gibco) supplemented with vitamins (Gibco), 10 % foetal bovine serum, 0.034 % sodium bicarbonate (J.T. Baker), and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin) (Sigma) at 35 °C in a CO_2-free incubator (Lab Line). This high-temperature-adapted cell line allows a shorter replicative cycle of DENV than in the wt C6/36 cells.

For plaque assays, BHK-21 cells were grown on glass Petri dishes in MEM (Gibco) supplemented with 0.034 % sodium bicarbonate (J.T. Baker), 10 % foetal bovine serum, and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin) (Sigma) at 37 °C in an incubator (Lab Line) with 5 % CO_2.

The DENV 2 New Guinea C strain (NGC) was kindly donated by InDRE (Instituto Nacional de Diagnóstico y Referencia Epidemiológicos, México) and propagated in newborn mice as described by Gould and Clegg [17] in order to obtain stocks with high viral titres suitable for further experiments [18]. Aliquots were stored at -80 °C (Sanyo Vip Series MDF-U53VC).

Persistently infected cell line

A persistently infected C6/36 cell line was developed following the protocol reported by Igarashi [14] with some modifications. C6/36 cells were detached with a scraper (Corning) and transferred to a 50-ml conical tube. The cells were then counted in a Neubauer chamber and centrifuged at 168 x *g* at room temperature for 10 min in a tabletop Eppendorf centrifuge (5804 R). The supernatant was removed, and the cell pellet was resuspended in 5 ml of culture medium containing DENV 2 (MOI of 0.1). The cells were incubated for 2 h at 37 °C with gentle shaking to allow virus entry and then transferred to a 75-cm^2 culture flask (Corning). The infection was allowed to progress for 7 days at 35 °C. The cells were detached as described previously, and half of the culture was transferred to a new flask every week. Half the volume of the culture medium was replaced at each transfer. The cytopathic effect (CPE) was monitored by light microscopy (Nikon Eclipse TE300 microscope), and the viral titres were determined by plaque assay as described below. The cells were frozen in liquid nitrogen at various passages.

Acute infection with DENV 2

C6/36 cell monolayers at 80 % confluency were infected with DENV 2 at an MOI of 0.2 for 1 h at 37 °C with gentle shaking. The monolayer was then washed twice with culture medium, 15 ml of fresh medium was added, and the infection was allowed to proceed for 48 h at 35 °C. These infected cells were used as a positive control.

Plaque assay

BHK-21 cells were seeded in a 24-well plate (Corning; 2.5×10^5 cells per well). The supernatants of the persistently infected cells were serially diluted (from undiluted to 1:10,000) in Hank's buffer supplemented with 0.5 % foetal bovine serum and incubated with BHK-21 cells for 4 h at 37 °C. Overlay medium (0.5 ml; MEM supplemented with 7.5 % foetal bovine serum, 1 % carboxymethylcellulose, and antibiotics) was then added, and the cells were incubated for 5 days at 37 °C in a CO_2 incubator. Finally, the medium was removed, and the monolayers were stained with naphthol blue black (0.1 % naphthol blue black, 0.165 M sodium acetate, and 6 % acetic acid) for 15 min at room temperature. The wells were washed with water, and the plaques were counted.

RNA purification

Total RNA was purified using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. The

RNA was then treated with 10 U of DNase I (Roche) for 15 min at room temperature. The nuclease was inactivated at 80 °C for 20 min, and the RNA was precipitated with 2.5 volumes of ethanol (J.T. Baker) and a 1/10 volume of 3 M sodium acetate (Sigma). The RNA was quantified in a Beckman spectrophotometer (DU 650).

Amplification of actin mRNA

The integrity of the RNA was verified by a single-step RT-PCR reaction using an RT-PCR Access kit (Promega) and 50 pmol of each primer. The primers were designed from the actin gene of *Aedes aegypti* (GenBank accession number U20287). The primer sequences were as follows: 5'-ACGTGAAATCGT TCGTGACATTAAG-3' (forward) and 5'-TTAACTTAGAAGCACTTGCGGTGAA-3' (reverse). The reaction was performed using a Mastercycler (Eppendorf) under the following conditions: one cycle at 48 °C for 60 min; one cycle at 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 55 °C for 1 min, and 68 °C for 1 min; and finally, one cycle at 68 °C for 10 min. The amplification products were visualised using electrophoresis in a 2 % agarose (Invitrogen) gel in TBE buffer (891.5 mM Tris, 88.9 mM boric acid and 249.8 mM EDTA) and stained with ethidium bromide (Bio-Rad).

Amplification of the viral genome

To analyse the DENV genome, 22 pairs of oligonucleotides (11 forward and 11 reverse primers) were designed based on the sequence reported for the DENV 2 NGC strain in GenBank (accession number AF038403; Table 1). One microgram of total RNA from the persistently infected, the uninfected (negative control), or the acutely infected (positive control) C6/36 cells was used as a template. The reactions were performed in a Mastercycler (Eppendorf) using a single-step RT-PCR Access kit (Promega) under the following conditions: one cycle at 48 °C for 1 h; one cycle at 94 °C for 4 min; 35 cycles at 94 °C for 30 s, a variable annealing temperature for 1 min, and 68 °C for 1 min; and finally, one cycle at 68 °C for 10 min. The annealing temperature was 60 °C for primers 3-4, 5-6, 9-10 and 15-16 and 55 °C for primers 1-2, 7-8, 11-12, 13-14, 17-18, 19-20 and 21-22. For the reaction shown in Fig. 2a, primers D1 (forward) and TS1 (reverse) (Table 1) were used under the following conditions: one cycle at 48 °C for

Table 1 Primers used for RT-PCR reactions

Forward primers	Reverse primers	Product size expected	Region amplified
DV2M1	**DV2M2**	**1,038 bp**	UTR 5'-C-prM-E
agtctacgtggaccgacaaagacag	tttttgccatcgtcgtcacacagct		
DV2M3	**DV2M4**	**1,020 bp**	E
acaaaccaacattggattttgaact	agctgtctccgaatggaggttctgc		
DV2M5	**DV2M6**	**959 bp**	E-NS1
acatcatcataggagtagagccggg	tccaataacccatatcggcatggac		
DV2M7	**DV2M8**	**1,019 bp**	NS1-NS2A
tagaaagtgcactcaatgacacatg	ccgctttctgctgtgaggatgttaa		
DV2M9	**DV2M10**	**1,019 bp**	NS2A-NS3
attggataccattagcattgacgat	tgtcatcttcgatctctggattgtc		
DV2M11	**DV2M12**	**960 bp**	NS3
tttttcgaaagagaaaattgaccat	caggtgtgttgatgttatctaggag		
DV2M13	**DV2M14**	**1,019 bp**	NS3-NS4A
aaggaatcattcctagcatgttcga	ctgtggcttggttggcaatagctgt		
DV2M15	**DV2M16**	**1,019 bp**	NS4A-NS4B-NS5
tgttaatgggtcttgggaaaggatg	taaggactctgagtgttcgtcctgc		
DV2M17	**DV2M18**	**959 bp**	NS5
acttagtggaaaattggttgaacaa	actctaagaagcgtgctccaagcca		
DV2M19	**DV2M20**	**1,019 bp**	NS5
ttgaagccctaggattcttgaatga	cttccatccatgggttttcttgaat		
DV2M21	**DV2M22**	**677 bp**	NS5-UTR 3'
acaaaactccagtggaatcatggga	agaacctgttgattcaacagcacca		
D1	**TS1**	**118 bp**	C
tcaatatgctgaaacgcgcgagaaaccg	cgccacaagggccatgaacag		

Primers were designed using the reported sequence of the DENV 2 New Guinea C strain (GenBank accession number AF038403)

15 min; one cycle at 94 °C for 4 min; 35 cycles at 94° C for 30 s, 60 °C for 1 min, and 68 °C for 1 min; and one cycle at 68 °C for 10 min. The products were analysed in a 1 % agarose (Invitrogen) gel in TBE buffer (891.5 mM Tris, 88.9 mM boric acid, and 249.8 nM EDTA) and stained with ethidium bromide (Bio-Rad). For semi-quantitative RT-PCR, different amounts (from 1 μg to 10 ng) of total RNA from persistently infected C6/36 cells were subjected to a reaction with primers 15 and 16 as describe above, and the products were analysed in a 10 % acrylamide gel. Densitometry analysis was performed with ImageJ software (Ferreira T, Rasband W. Version IJ1.45, 2011).

Cloning and sequencing of the viral genome

The RT-PCR products were cloned into a pJET plasmid vector (Fermentas Inc.) according to the manufacturer's instructions. The products obtained with the primers 3-4, 5-6, 15-16, 17-18, 19-20, and 21-22 were purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN) and in some cases were re-amplified by PCR (see results). Only four RT-PCR reactions (those produced with primers 5-6, 15-16, and 19-20) resulted in products in addition to the expected 1-kb band. They were also purified and cloned as described above, and each of the plasmids was used to transform competent DH5α cells using a heat shock protocol. The cells were grown on LB-ampicillin (100 μg/ml) agar plates, and the plasmids from selected colonies were purified using a Zyppy™ plasmid miniprep kit (Zymo Research), cut with restriction enzymes and sequenced using an ABI-3100 sequencer (Facultad de Estudios Superiores-Iztacala and Instituto de Fisiología Celular of Universidad Nacional Autónoma de México) by the Big Dye Terminator fluorescence method. The sequences were visualised using BioEdit software (version 7.0.9.0, Hall, TA 1999 Nucl. Acids Symp. Ser. 41:95-98) and analysed using CLUSTALW (Biology Work Bench version 3.2, San Diego Supercomputer Center http://workbench.sdsd.edu), Translate and Compute pI/Mw (ExPASy proteomics server, Swiss Institute of Bioinformatics http://us.expasy.org), and BLAST (National Institute for Biotechnology Information http://www.ncbi.nlm.nih.gov) software.

Detection of the NS1 protein

Uninfected or persistently infected C6/36 cells were seeded in a 24-well culture plate (5×10^5 cells per well) and incubated overnight at 35 °C. Three wells were infected with DENV 2 at an MOI of 0.2 as described previously, and the remaining wells were incubated with culture medium alone. After 48 h of infection at 35 °C, the supernatant was removed, and the monolayers were washed three times with PBS and lysed by freeze-thawing three times. Cellular debris was removed by centrifugation, and the presence of NS1 in the supernatant was determined using a Platelia™ Dengue NS1 AG test (Bio-Rad) according to the manufacturer's instructions. The assay was read in a Stat Fax 142510 ELISA reader at 450/620 nm. A similar assay was performed with the supernatants obtained from the immunofluorescence assay (see next section).

Immunofluorescence assay

C6/36 cells (0.4×10^5 cells per well) were grown in a Chamber (Lab-Tek) for immunofluorescence and incubated at 35 °C for 24 h. The culture medium was removed, and the monolayers were incubated for 1 h at 37 °C with shaking with 200 μl of the supernatants of the uninfected cells, the cells acutely infected with DENV 2 or those persistently infected with DENV 2. After extensive washing, fresh media was added, and after incubation for 1 (24 h) or 5 days at 35 °C, the cell monolayers were processed for immunofluorescence. The supernatants were analyzed by Platelia assay (see above). The C6/36 monolayers were fixed with PBS-4 % paraformaldehyde (MP Biomedicals) for 30 min at room temperature. They were then washed four times with PBS and blocked with PBS-BSA 1 % overnight at 4 °C. The cells were washed three times with PBS and incubated overnight at 4 °C with a mouse polyclonal antibody anti-DENV (MS X Dengue Complex MAB8705, Chemicon international) diluted 1:200 in PBS. After four washes with PBS, the monolayers were incubated for 2 h at room temperature with a FITC-conjugated anti-mouse antibody (FITC-goat anti-mouse IgG 62-6511, Invitrogen) diluted 1:500 in PBS and protected from light. The cells were washed four times with PBS, and the nuclei were stained with 2 μl of vectashield-4',6-diamidino-2-phenylindole (Vector Laboratories). Finally, the cells were observed under an Eclipse 80i immunofluorescence microscope (Nikon).

Western blot assays

Total protein extracts were obtained from uninfected C6/36 cells, those infected for 48 h or those persistently infected as described previously [19]. The extracts were subjected to 10 % SDS-PAGE (Mini-PROTEAN system, Bio-Rad) and transferred to a nitrocellulose membrane using a Trans-Blot semi-dry transfer cell (Bio-Rad) in 48 mM Tris, 39 mM glycine, and 20 % (vol/vol) methanol. The transfer efficiency was determined by staining with 0.1 % Ponceau red in 7 % trichloroacetic acid. The membrane was blocked with gentle shaking in 5 % casein in PBS for 2 h at room temperature and then incubated overnight at 4 °C in

primary antibody diluted in PBS. The membrane was washed three times with PBS-0.5 % Tween 20 and incubated with secondary antibody diluted in PBS at room temperature for 2 h. After washing with PBS-0.5 % Tween 20, the reaction was developed using a chemiluminescence kit (SuperSignal West Pico, Thermo Scientific) according to the manufacturer's protocol and exposed to MXB film (Kodak).

The rabbit anti-NS5 antibody was kindly donated by Dr. Padmanabhan and diluted 1:1,000 or 1:5,000. The mouse monoclonal anti-E antibody (Abcam 80914), which recognises amino acids 52 to 280 of the DENV 2 E protein, was diluted 1:2,000. The HRP-coupled secondary antibodies used were anti-mouse (Zymed) at a 1:20,000 dilution and anti-rabbit (Invitrogen) at a 1:10,000 dilution.

Results

Generation of a C6/36 cell line persistently infected with DENV type 2

Previous studies have shown that DENV can persistently infect various cell lines [14, 15, 20, 21], and the infections might be maintained by defective viruses [14, 15]. However, the sequences of the defective viral genomes have not been completely determined [8]. To analyse the persistence of the infections and to sequence the defective viral genomes, a persistently infected C6/36 cell line was generated by infecting monolayers of C6/36 cells (*Aedes albopictus*) [12] adapted to grow at 35 °C [16] with DENV 2 following an approach reported previously by Igarashi [14]. Since the persistently infected C6/36 cells obtained using this protocol displayed oscillating virus titres, the generation of thermosensitive and neurovirulent attenuated virus mutants, and homologous viral interference – all evidence of the presence of defective viruses [14] – we selected this protocol to generate our persistent infected cell line. The C6/36 cell line has been used extensively as a model for infection of mosquitoes by DENV [14, 15, 22–30], flaviviruses [11, 20], and other viruses [24–28].

Over a period of 50 weeks during which the culture media was partially replaced every week, the cells were observed microscopically, and the viral titre was determined by plaque assay in BHK-21 cells.

As shown in Fig. 1a, the viral titre fluctuated during the 32-week period, with two main peaks occurring at weeks 7 and 20. The titre then decreased progressively until no virus was detected in the culture media at week 42. The average plaque size from the parental DENV 2 was 1.4 mm, whereas the plaques of the virus released from the persistently infected cells were slightly reduced in size to 0.9 mm at week 6. At week 9, it was evident that the plaques varied in size, and after this point, the diameter of the plaques decreased to 0.7-0.9 mm (data not shown). CPE was clearly observed during the first 7 weeks (Fig. 1b), but it gradually diminished after this time and was completely absent at week 30. At all times, the cells grew normally.

To determine if the viral genome was present in the cells after week 42, when neither a viral titre nor CPE was detected, RT-PCR using total RNA and specific primers for

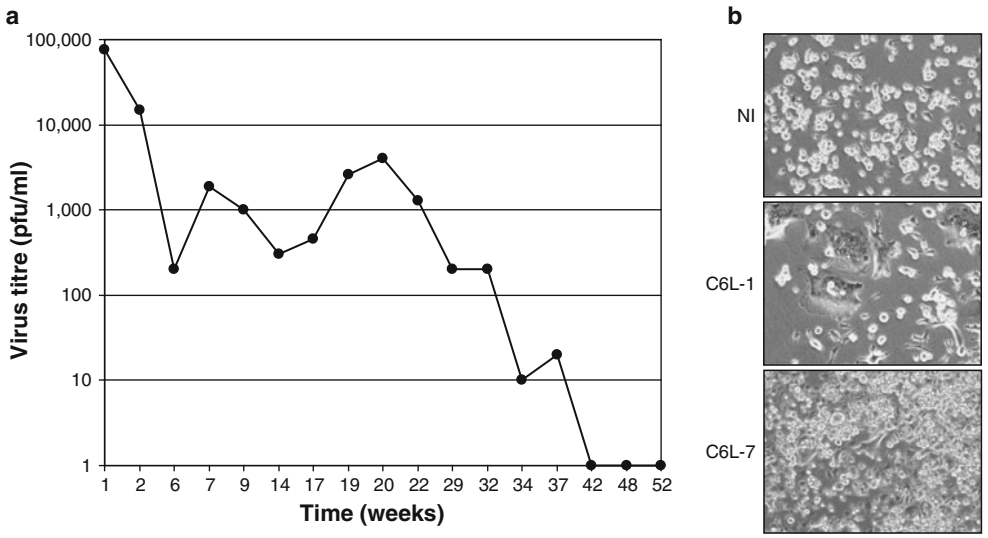


Fig. 1 Establishment of a persistently infected cell line. C6/36 cells were infected with DENV 2 (NGC strain) at a MOI 0.1, and every week, the culture media was partially changed. **A.** Virus titers of the supernatants of persistently infected cells determined by plaque assay in BHK-21 cells. **B.** Microphotographs of the persistently infected cell line at different times postinfection: 1 (L-1) and 7 (L-7) weeks. NI, uninfected C6/36 cells

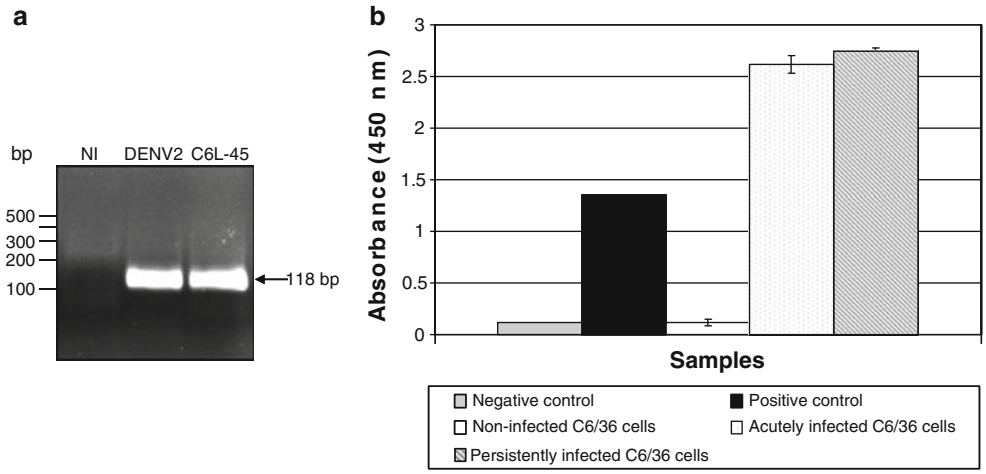


Fig. 2 Detection of DENV in persistently infected C6/36 cells. **a** 2 % agarose gel stained with ethidium bromide showing an RT-PCR reaction performed with total RNA from C6/36 cells and primers for the C region of the virus genome. The 100-bp molecular marker ladder (New England Biolabs) is shown at the left side of the gel. NI, non-infected C6/36 cells; DENV 2, C6/36 infected with DENV 2 for 48 h; C6L-45, persistently infected C6/36 cell line (passage 45). **b** Detection of NS1 protein using a Platelia NS1 AG kit; the positive and negative controls included in the kit are shown. The assay was performed in triplicate, and the SD values are shown

the C region was performed. A 118-bp product was amplified in both the positive control cells (C6/36 cells acutely infected with DENV 2) and persistently infected cells (harvested at week 45), but not in the uninfected cells (Fig. 2a). Similar RT-PCR reactions were performed using cells from other passages, and the viral genome was always detected (data not shown). Finally, the expression of the viral NS1 protein was evaluated in C6/36 cells using a Platelia NS1 AG kit (Bio-Rad). The presence of NS1 was detected as expected in acutely infected as well as persistently infected cells (Fig. 2b), suggesting that at least part of the viral genome was translated in the persistently infected cells. Taken together, these results indicate that we were able to generate a cell line persistently infected with DENV 2.

To confirm that virus particles were released from the persistently infected cells, an immunofluorescence assay was performed. The culture medium recovered from the persistently infected cells after a 72 h of incubation at 35 °C was used to infect regular C6/36 cell monolayers for 1 h at 37 °C. The presence of the virus in the cells was confirmed by an immunofluorescence assay using an anti-dengue antibody and an FITC-coupled secondary antibody. The presence of NS1 in the supernatants of the re-infected cells was determined again using the Platelia NS1 AG kit. The supernatants of the uninfected and acutely infected cells were used in control assays. Twenty-four hours after infection, the immunofluorescence signal in the C6/36 cells infected with the supernatant of the persistently infected cells was barely detectable (see Fig. 3a, C6-L, 24 hours), but the NS1 protein was clearly present in the culture medium (Fig. 3b). These results indicate that the infectious virus particles were released from the persistently infected cells, but apparently at a very low rate. Interesting, if the infection was allowed to progress for 5 days, the levels of NS1 protein released into the culture medium were similar to those of NS1 released by the cells infected with the supernatant of acutely infected cells (see Fig. 3b), and the fluorescence signal was clearly visible (Fig. 3a, C6-L, 5 days).

Detection of defective viral genomes

Several studies using flaviviruses have demonstrated the presence of defective viral genomes in persistently infected cell lines [10, 13, 29]. To detect defective DENV 2 genomes in our persistently infected C6/36 cell line, an approach similar to that of Lancaster et al. [29] was used. Briefly, the total RNA of the C6/36 cells persistently infected with DENV 2 was purified and treated with DNase I, and RT-PCR reactions were performed with 11 pairs of specific oligonucleotides to screen for the complete DENV genome (see Table 1). RNA from uninfected C6/36 cells and from acutely infected (48 h) C6/36 cells was used as the negative and positive controls, respectively. The PCR products were analysed by electrophoresis in a 1 % agarose gel (data not shown). Using this approach, we were able to detect defective genomes at passage 48, but they were more easily detected at passage 60. Furthermore, we selected this passage for further experiments.

The reactions using the template from persistently infected cells, in which additional bands were detected (Figs. 4, 6), were further analysed; three PCR products that corresponded to the E and NS5 coding sequences showed evidence of possible deletions.

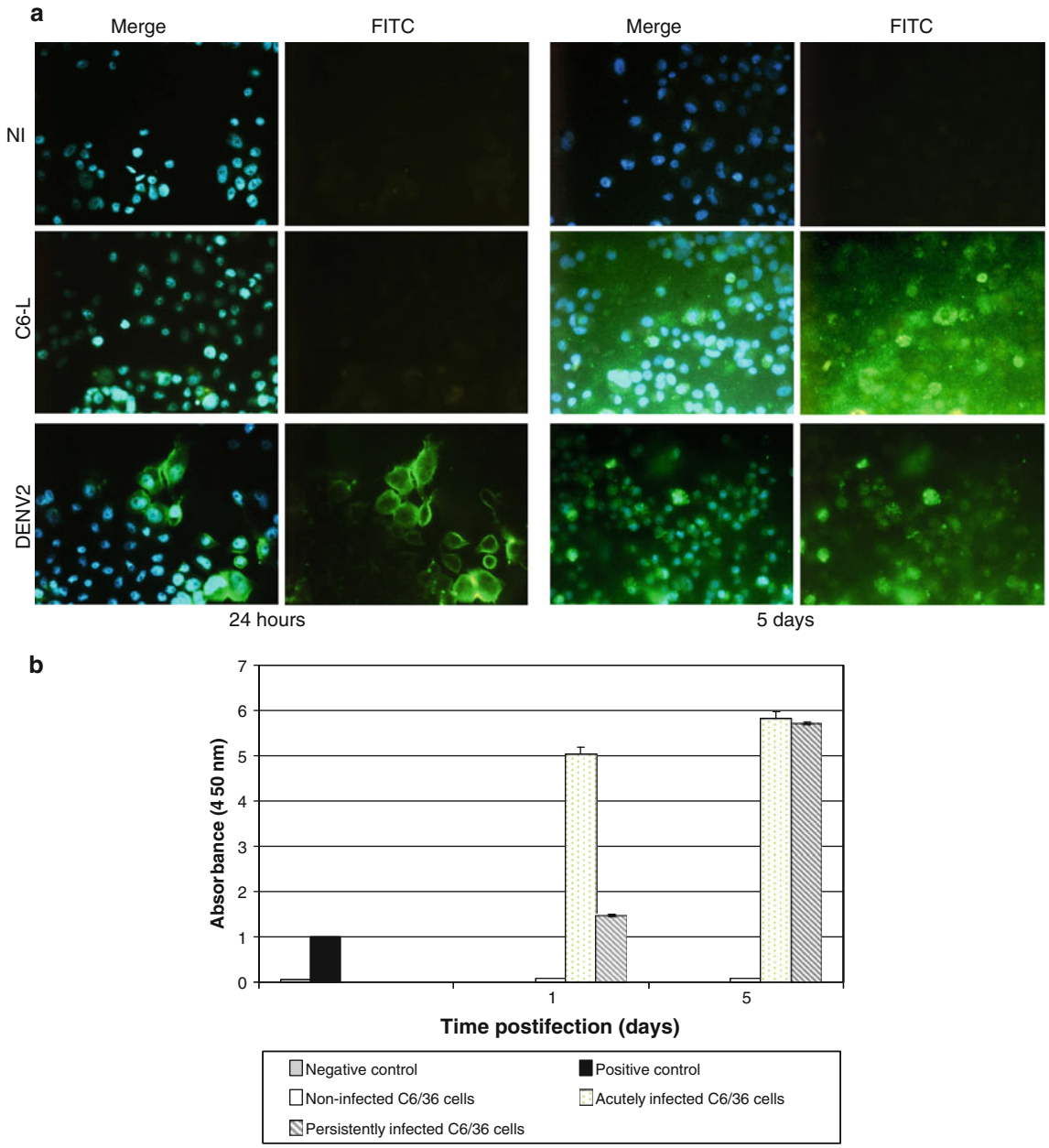


Fig. 3 Detection of virus particles in supernatants. Monolayers of C6/36 cells seeded in an immunofluorescence chamber were incubated with supernatants obtained from C6/36 acutely or persistently infected with DENV 2 and from uninfected cells for 1 h at 37 °C with shaking. Then, the inoculum was removed, and the monolayers were extensively washed. After 1 (24 h) or 5 days, the cells were processed for immunofluorescence, and the supernatant was recovered to determine the presence of NS1 protein by Platelia assay. **A**. Immunofluorescence assay of C6/36 cells incubated with supernatants obtained from non-infected cells (NI) and cells persistently infected (C6-L) or acutely infected with DENV 2 (DENV 2). The assay was performed 24 h or 5 days postinfection. The virus was detected using a primary anti-DENV antibody and a secondary FITC-coupled antibody. The nuclei were stained with DAPI. **B**. Detection of NS1 protein in the supernatants of the immunofluorescence assay using a Platelia NS1 AG kit; the positive and negative controls included in the kit are presented. The assay was performed in triplicate and the SD values are shown

E protein

When RT-PCR reactions were performed with primers 1-2 (data not shown) and 5-6 (Fig. 4b), an approximately 1-kb product was observed in both the acutely and persistently infected C6/36 cells, suggesting that there were no deletions within these regions. However, when primers 3-4 were used with the RNA from the persistently infected cells, a band of approximately 600 bp was detected in addition to the expected 1-kb product, indicating the possible presence of a deletion (Fig. 4a).

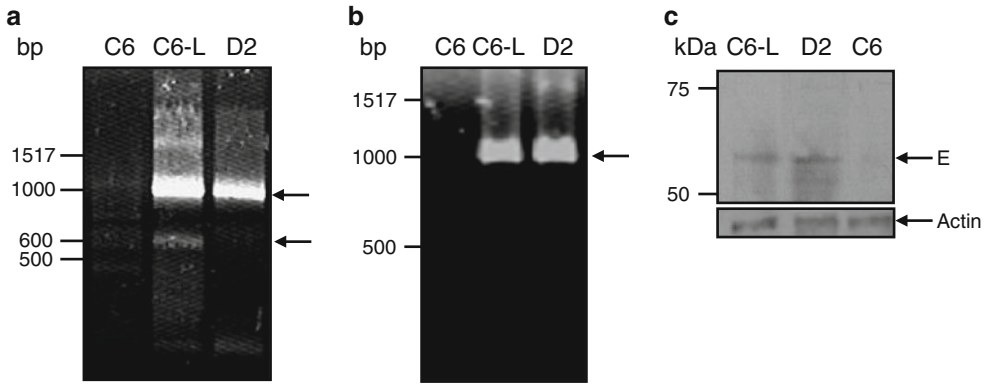


Fig. 4 Analysis of the coding region of the E protein. RT-PCR using total RNA from uninfected C6/36 cells (C6), and cells acutely (D2) or persistently infected (C6-L) with DENV 2. Reactions were performed with primers 3 and 4 (**a**) or 5 and 6 (**b**). The amplification products are indicated by arrows. The molecular markers (100 bp DNA Ladder, New England Biolabs) are indicated at the left side of each gel. **c** Western blot assay using a monoclonal anti-E-protein antibody. The E protein is indicated by an arrow. The markers (Bio-Rad) are indicated at the left side of the gel

All of the products of the reactions performed with primers 3-4 and 5-6 in either the acutely or persistently infected cells were purified, cloned into the pJET vector, and sequenced. The sequences obtained were compared using BLAST software and then translated into amino acids using the Translate tool of the ExPASy proteomics server. The amino acid sequences, which included the NGC DENV 2 reference strain reported in GenBank, were compared using the CLUSTALW software, and the results are shown in Fig. 5.

From the C6/36 cells acutely infected with DENV 2, two clones were obtained and sequenced, and a representative clone is displayed in Fig. 5 (DENV-2). When the sequence of the E protein was analysed, only the following four mutations were detected: N83I, E147K, V347A and I402F. These mutations were localised in domains II, I, III, and the transmembrane region, respectively. Except for E147K, where a negatively charged amino acid was replaced with a positively charged amino acid, the other mutations were conserved (polar for polar or hydrophobic for hydrophobic), suggesting that these mutations would not have a deleterious effect on the E protein. Interestingly, the four mutations reverted to wild type in the persistently infected cells (see Fig. 5, clone 29).

In the persistently infected cells, two identical clones were obtained from the cloning of the 1-kb fragments. The sequence, identified as "29" in Fig. 5, contained only three mutations (V114A and E202G in domain II and K393R in domain III). Whereas two mutations (V114A and K393R) resulted in a conservative change (hydrophobic for hydrophobic and positively charged for positively charged), the E202G mutation substituted a negatively charged amino acid for a polar amino acid. These results further suggested that there were no major modifications made to the sequence of the E protein in the persistently infected cells.

Finally, from the 600-bp fragment, two identical clones were obtained. The sequence identified by number 21 in Fig. 5 exhibited 68 mutations; 30 mutations were located in domain I (44 %), 37 in domain II (54 %) and only one in domain III. Overall, 31 were conserved, 13 were non-equivalent, and 24 represented a change in the charge of the amino acid. Although mutation I113V was located at the end of the fusion peptide (see Fig. 5, clone 21), both valine and isoleucine are hydrophobic amino acids. Thus, this mutation did not modify the hydrophobicity of this region. The most important modification in clone 21 was the deletion of 147 residues from amino acids 222 to 368, which affected all three domains of the protein, particularly domain III, as much as 73 % of the domain was deleted (see Fig. 5, clone 21, dashed line).

Interestingly, in all of the viral genomes analysed and in the cells that were acutely or persistently infected, the glycosylation sites, the fusion peptide, and the transmembrane domain were well-conserved, and when a mutation occurred, the amino acid change was equivalent.

To confirm the deletion detected by RT-PCR in the E protein sequence, a western blot of the total protein extracts from acutely and persistently infected cells was performed. The protein extracts from the uninfected cells were used as a negative control. An anti-E protein antibody detected a protein of approximately 55-60 kDa in both the acutely and persistently infected cells, which correlated with the size of the E protein in DENV 2 and clone 29 (Fig. 4c). Using the Compute pI/Mw software (ExPASy proteomics server, Swiss Institute of Bioinformatics), the molecular weight of the protein encoded by clone 21 was calculated as 37,878.89 Da. Unfortunately, after several attempts, we

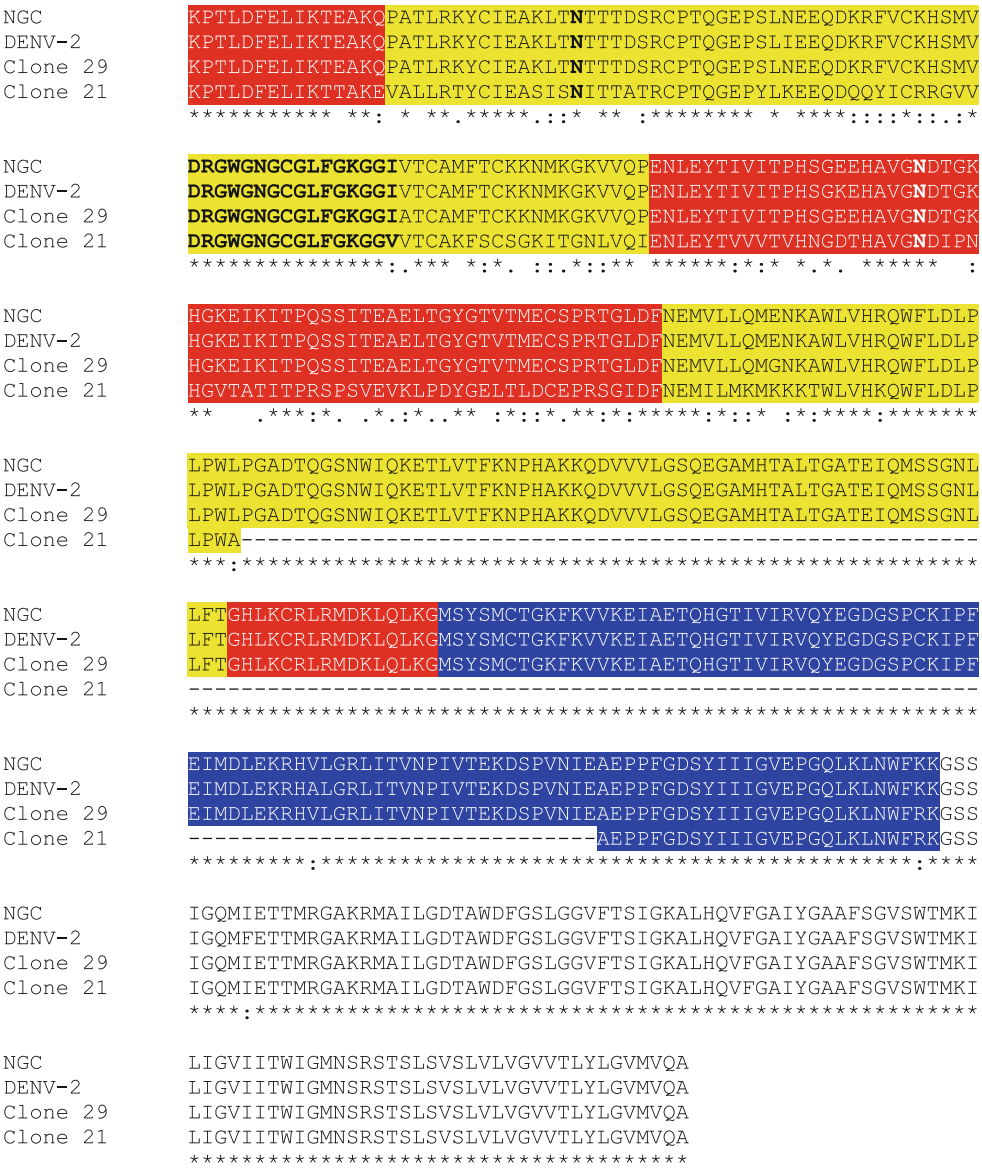


Fig. 5 Amino acid sequence alignment of the E protein coding region. The amplification products obtained with primers 3-4 and 5-6 were purified, cloned in the pJET vector and sequenced. The amino acid sequence was obtained with the Translate software (ExPASy Proteomic Server, Swiss Institute of Bioinformatics) and aligned using the CLUSTALW software (San Diego University, California, USA). DENV-2 denotes the sequence obtained from C6/36 cells acutely infected with DENV 2 and clones 29 and 21 from persistently infected C6/36 cells. The sequence of DENV 2 (NGC strain) was included as a reference (GenBank accession number AF038403). Domains I (red), II (yellow) and III (blue) are colored according to Modis *et al.* [35]. The two glycosylation sites and the fusion peptide are indicated in **bold**. Fully conserved (*), strongly conserved (:), and weakly conserved (.) residues as well as no consensus (blank) amino acids are indicated by the corresponding symbols. A dashed line indicates the deletion present in clone 21

were unable to detect this truncated version of the E protein in the persistently infected cells by western blot.

NS5 protein

In another region of the viral genome, some modifications were detected within the NS5 coding region. When primers 15 and 16 were used in an RT-PCR reaction with total RNA, an expected product of 1,019 bp was obtained in the acutely and persistently infected cells (Fig. 6a), but another product between 500 and 600 bp was also amplified only from the RNA of the persistently infected cells (Fig. 6a, C6-L). A similar result was obtained when primers 19 and 20 were used, but in this case, the molecular weight of the additional band was approximately 350 bp (Fig. 6b, C6-L). Although primers 19-20 amplified a nonspecific product of approximately 500 bp, usually when the RNA of the uninfected cells was used as the template, the 350-bp product was only detected using the RNA of the persistently infected cells as a template (Fig. 6b). The reactions performed with primers 17-18 and 21-22 only generated products at the expected sizes (Table 1 and data not shown). To confirm the identities of the fragments obtained in the RT-PCR reaction using primers 19 and 20, the two 1,019-bp bands and the 350-bp band displayed in Fig. 6b were excised from the gel, purified using a QIAquick Gel Extraction Kit (QIAGEN), and used as templates for PCR reactions with the same primers. The products were analysed by agarose gel electrophoresis. In all cases, fragments of the expected sizes were obtained in the absence of additional bands (Fig. 6c), indicating that both bands were the amplification products of primers 19 and 20.

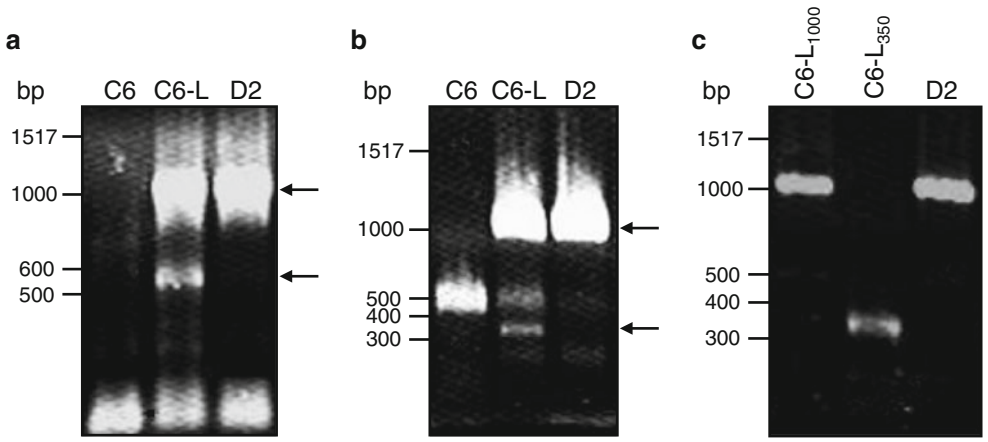


Fig. 6 Analysis of the coding region of the NS5 protein. RT-PCR using total RNA from uninfected C6/36 cells (C6) acutely (D2) or persistently infected (C6-L) with DENV 2. Reactions were performed with primers 15 and 16 (**a**) or 19 and 20 (**b**). The amplification products are indicated by arrows. **C**. PCR re-amplification products from bands purified from B; C6-L1000 and C6-L350 are 1000- and 350-nt bands, respectively, from persistently infected cells; D2, product of 1000 bp from acutely infected cells. The molecular markers (100 bp DNA Ladder, New England Biolabs) are indicated at the left side of each gel

All of the products obtained from the RT-PCR reactions performed with primers 15-16, 17-18, 19-20, and 21-22 were purified, cloned into the pJET vector and sequenced. The sequences were used to construct the complete NS5 sequence. The NGC strain reported in GenBank was used as a reference (see Fig. 7, NGC). Seven clones were obtained from the products of the RT-PCR reactions performed with RNA from the acutely infected cells, and the translations of representative sequences are shown in Fig. 7 (DENV-2). Twenty-five mutations were found, 4 in the methyltransferase domain and 21 in the RNA polymerase domain. Six mutations were conserved, and one, the R326K mutation, was located in the nuclear localisation signal (NLS) of NS5. With the exception of one mutation, all of the mutations detected in the RNA of the acutely infected cells reverted to the wild-type sequence in the viral genomes present in the persistently infected cells. Ten different clones were obtained from the RT-PCR products of the persistently infected cells. Seventy mutations were detected, 50 % of which were in the methyltransferase domain, and the remaining 50 % were in the RNA polymerase domain. Thirty of the mutations (43 %) were conserved, and some of the clones contained one or several stop codons. Interestingly, all of the RT-PCR products that were approximately 1 kb in size had sequences similar to that of the the New Guinea reference strain (see Fig. 7, C6L_Wt). Only seven mutations were detected, and four of these mutations (57 %) were conserved, including one located in the NLS.

Three clones were obtained using primers 15-16 and the RNA of the persistently infected C6/36 cells. Sequencing within the 600-bp fragment revealed a major deletion of 153 amino acids (from residues 4 to 156) of the methyltransferase domain. Importantly, this deletion affected the hydrophobic pocket of the domain.

Similarly, within a 350-bp fragment generated using primers 19-20 and sequenced within three clones, a longer deletion of 228 amino acids (from residues 597 to 825) of the RNA polymerase domain was detected. This deletion affected the NTP catalytic domain (GDD) and the GTP-binding sites as well as the zinc-binding pockets.

To correlate the deletions detected in the viral genome with protein expression, a western blot was performed using a polyclonal anti-NS5 antibody. A band at the expected size of 102-103 kDa was observed in the protein extracts of both the acutely and persistently infected cells (Fig. 8a). The size of this band correlated with the sequences from DENV-2 and C6L_Wt obtained in the RT-PCR analysis. Using the Compute pI/Mw software (ExPASy proteomics server, Swiss Institute of Bioinformatics), the molecular weights of the predicted NS5 proteins, C6L_[15/16] and C6L_[19/20], were calculated to be 86,211.21 and 77,406.21 Da, respectively. We could only detect these predicted products using a less dilute solution of the polyclonal anti-NS5 antibody (1:1,000, see Fig. 8b). Among other bands, the polyclonal antibody detected a faint band below the 103 kDa product (likely the predicted 86-kDa C6L_[15/16] protein) and a stronger band of approximately 75 kDa (likely the predicted 77 kDa C6L_[19/20] protein) only in the protein extracts of the persistently infected C6/36 cells (Fig. 8b, line C6-L, indicated with asterisks), suggesting that the two truncated versions of the NS5 protein were expressed in the persistently infected cells but apparently in a lower rate than the wild-type protein.

Finally, in order to know the relative amounts of wild-type versus defective viral genomes, a semi-quantitative RT-PCR was performed using decreasing amounts of total RNA from persistently infected cells and primers 15 and 16, where the viral defective genome was more evident. The

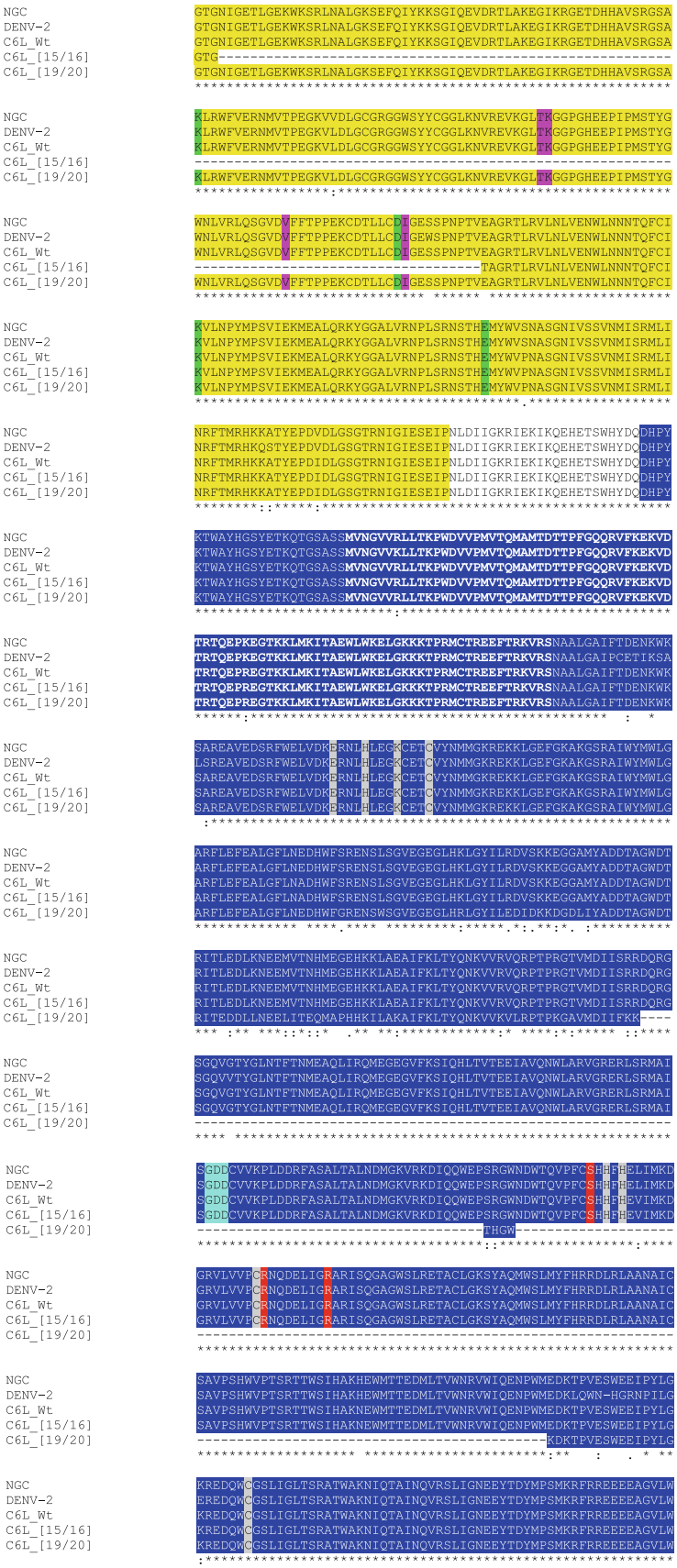


Fig. 7 Amino acid sequence alignment of the NS5 protein coding region. The amplification products obtained with primers 15-16, 17-18, 19-20, and 21-22 were purified, cloned in the pJET vector and sequenced. The amino acid sequence was obtained with the Translate software (ExPASy Proteomic Server, Swiss Institute of Bioinformatics) and aligned using the CLUSTALW software (San Diego University, California, USA). DENV-2 denotes the sequence obtained from C6/36 cells acutely infected with DENV 2; and C6L, from persistently infected C6/36 cells. The sequence of the DENV 2 (NGC) strain was included as a reference (GenBank accession number AF038403). The methyltranferase and RNA polymerase domains are colored in yellow and blue according to Egloff *et al*. [39] and Yap *et al*. [43], respectively. The overlapping region of the two domains is indicated in white [43]. The catalytic tetrad and the hydrophobic pocket of the methyltransferase domain are indicated in green and pink according to Egloff *et al*. [39] and Zhou *et al*. [40], respectively. **Bold** letters indicate the NLS [46, 47]. The NTP catalytic and binding site, the zinc binding pockets and the GTP binding site [43] are indicated in light blue, gray and red respectively. Fully conserved (*), strongly conserved (:), and weakly conserved (.) residues as well as no consensus (blank) amino acids are indicated by the corresponding symbols. Dashed lines indicate deletions

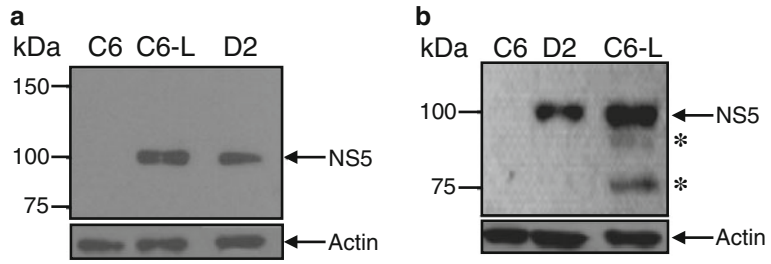
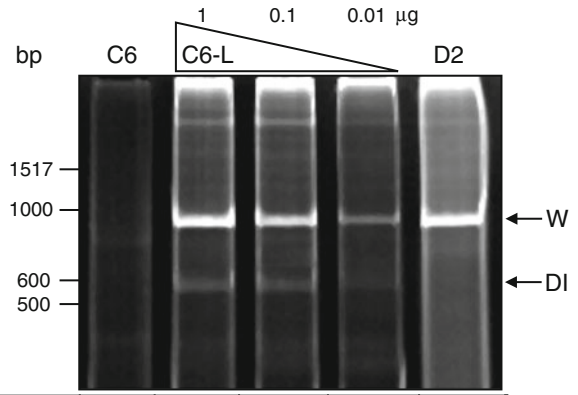


Fig. 8 Western blot assay for NS5 protein. Total protein extracts from uninfected (C6) and persistently (C6-L) or acutely infected (D2) cells with DENV 2 were subjected to SDS-PAGE and transferred, and the viral NS5 protein was detected using a polyclonal anti-NS5 protein antibody diluted 1:5000 (**a**) or 1:1000 (**b**). The NS5 protein is indicated by an arrow. * indicates the additional bands detected in the protein extract from the persistently infected cells. The markers (Bio-Rad) are indicated at the left side of each gel



W	3282	40884	32171	12430	42453
DI	2703	9133	8480	ND	ND
Ratio W/DI	**ND**	**4.47**	**3.79**	**ND**	**ND**

Fig. 9 Semi-quantitative RT-PCR assay. RT-PCR using different amounts of total RNA (indicated at the top of the gel) from C6/36 cells persistently infected with DENV 2 (C6-L) was performed with primers 15 and 16 and analysed in a 10 % polyacrylamide gel. Reactions performed with RNA from uninfected cells (C6) and cells acutely infected with DENV 2 (D2) were included as controls. The amplification products of both wild-type (W) and defective viral genomes (DI) are indicated by arrows. The molecular markers (100 bp DNA Ladder, New England Biolabs) are indicated at the left side of the gel. The result of a densitometry analysis performed with ImageJ softaware is shown below the gel. ND, non-determinate

wild-type:defective genome ratio was determined by densitometry analysis. As can be seen in Fig. 9, the relative abundance of wild-type viral genome was approximately four times more than that of the defective RNA, and when 10 ng of RNA was used in the RT-PCR reaction, the product corresponding to the defective genome was no longer visible.

Discussion

Persistent DENV infections occur naturally in mosquitoes [5] and in mosquito cell cultures [14, 15, 21]. During this type of infections in cell cultures, important changes in the viral replicative cycle are observed. Those changes are often associated with reductions in viral yield and CPE as well as in the presence of defective interfering particles. However, several aspects of viral persistence are still not understood [30].

Flaviviruses can establish a persistent infection in various cell types, especially those of arthropod origin [11, 13–15, 30, 31]. In this study, we generated a C6/36 cell line persistently infected with DENV 2 according to a protocol reported by Igarashi [14] with some modifications. As reported previously, we observed several changes in the characteristics of the culture, including fluctuating viral titres [14, 15], a progressive decrease in plaque size as well as a mosaic pattern of varying plaque sizes during the course of viral infection [11, 15, 30], and reduction in CPE [15, 21, 31–33]. With regard to the viral proteins, reports vary. While several authors have observed a reduction in viral protein expression during persistent JEV infections [13, 32, 34], other authors have reported a large number of viral-antigen-positive cells during persistent infections with both JEV [31, 33] and DENV [20, 21]. In our persistently infected cell cultures, even though the virus was either absent or present at low levels, the expression level of the NS1 protein detected by the Platelia NS1 AG kit was similar to the expression level of NS1 in the acutely infected cells. Moreover, after 42 passages, the persistently infected cell cultures showed no virus production, at least when measured by plaque assay, but the viral genome was easily detected by conventional RT-PCR methods. We could not detect the release of virus particles by immunofluorescence when normal C6/36 monolayers were re-infected with supernatants of a persistently cell culture for 24 h. However, the more sensitive Platelia assay was clearly positive, indicating the release of infectious particles, likely in very low amounts. Interestingly, the infection pattern was quite similar to that of the positive control when it was allowed to progress for 5 days. This result suggests that the virus particles released by the persistently

infected C6/36 cells are able to establish a productive infection after several replicative cycles in new cells. If DIs are generated, they are not be able to block the infection by the parental virus, indicating that the effect observed in the persistently infected cells is likely due to an intracellular mechanism.

Defective viral genomes are the truncated versions of a parental genome arising from aberrant events that occur during the replication and/or transcription of the virus, and the viral polymerase has been implicated in these aberrant events (reviewed in ref. 7). The defective viral genomes have been detected in several viral systems. Because they possess the genomic elements required for viral replication, they can be copied and participate in the establishment and maintenance of persistent infections [6]. DI particles have been identified and analysed in persistent infections by several flaviviruses, such as JEV [13, 32], SLE [11] and MVE [16]. Several reports have also suggested the presence of defective DENV genomes [14, 15] in persistently infected cells. However, there are only two reports that have confirmed their existence, and they were focused on acute infections [8, 9]. In this work, we first tried to detect the defective viral genome by northern blot hybridization using the biotinylated non-translated 3' region of DENV 2 as a probe (data not shown), but this was unsuccessful, probably because of the lack of sensitivity of this technique [9]. Therefore, we performed a complete screening of the DENV 2 genome present in persistently infected C6/36 cells using RT-PCR and specific oligonucleotides as reported previously [29].

We were able to detect defective genomes in passage 48, but they were more easily detected at passage 60. We did not perform experiments to detect them in early passages, but in previous work in which C6/36 cells were transfected with RNA from a DENV 2 infectious clone, subgenomic fragments were visualized at passage 4 [9].

Two sequences present in the E and NS5 proteins of DENV were found deleted in the viral genomes in persistently infected C6/36 cells.

E is the major envelope protein present in DENV, and it is a class II fusion protein that mediates both receptor binding and viral entry into cells. It carries the main antigenic determinants and has three domains, which are designated I (residues 1-52, 132-193 and 280-296), II (residues 52-132 and 193-280), and III (296-394); the protein also contains a transmembrane region, two glycosylation sites and a fusion peptide (located in domain II) that is implicated in viral fusion with the cell membrane during entry [35, 36]. In this report, the coding region for the E protein of genomes harvested from acutely and persistently infected cells was cloned, sequenced and compared with that of the NGC DENV 2 reference strain. In persistently infected C6/36 cells, two variants of the E coding region were found. One (clone 29) was very similar to the wild-type E protein coding region, with only three mutations, and its product was detected by western blot using a monoclonal anti-E protein antibody. The other (clone 21) was a defective version with 68 mutations, and only 45.6 % of the amino acids were conserved, but the most important finding was that this variant of the genome contained a deletion (about 147 amino acids in the protein product) that encompassed domains I and II, but mainly domain III (73 %). The latter domain has been implicated in virus-receptor interactions and is important for viral attachment and entry [35]. Deletions in the coding region of the E protein are a common finding and have been reported in other flaviviruses, such as JEV [10–12] and MVE [29].

The expected molecular weight of the E protein encoded by the defective genome is 38 kDa. A previous study reported the presence of a 27-kDa E protein in C6/36 cells infected with a DENV 1 circulating between mosquitoes and humans in Myanmar. This truncated version was the result of a stop codon at amino acid 248. Interestingly, the deletion in clone 21 was in the same region as that described previously [8]. Unfortunately, even after several attempts, we could not detect the truncated protein by western blot. This result can be explained in two ways: First, the antibody used in our experiments is monoclonal, and it recognises amino acids 52-280, located in domains I and II. The deletion in clone 21 comprises amino acids 222 to 368; thus, the overlapping region of approximately 58 residues absent in clone 21 could alter recognition of the E protein by the antibody. The second possibility is that while this defective genome is present in persistently infected cells, it may not be expressed, or its expression levels may be very low; thus, the protein would not be detected under the conditions used in our assay.

The other region of the viral genome in which deletions were detected was the NS5 coding region. NS5 is the largest protein (900 amino acids) encoded by the DENV genome, and it is implicated in viral replication and methylation [37, 38]. The NS5 protein has two main domains: the methyltransferase [38–41] and RNA-dependent RNA polymerase (RdRP) [42, 43] domains. The methyltransferase domain is at the N-terminus of the protein [38, 40] and is an AdoMet-dependent MTase responsible for the guanine N-7 and ribose 2'-O methylations during viral cap formation [38, 39]. The RdRP domain is larger and localised in the C-terminus of the protein. It contains a structure composed of "palm", "fingers" and "thumb" subdomains [43] and is responsible for the synthesis of new viral genomes [44]. Because the RdRP is able to interact with STAT2 and induce its degradation via the ubiquitin-proteasome pathway, the RdRP also plays an important role in the viral anti-interferon response [45]. Finally, the RdRP has a bipartite NLS α/β-β1 that mediates

translocation of NS5 to the nucleus, which is an important event in the inhibition of IL-8 production in mammalian cells [46, 47].

Although deletions in the NS5 protein sequence have not been reported in other persistent infections with flaviviruses, sequence analysis of defective viral genomes in cells infected with influenza virus [7] and vesicular stomatitis virus (VSV) [6] have identified deletions in polymerase genes. In our persistently infected C6/36 cells, three forms of NS5 were detected. One resembled the wild-type protein, and the other two were encoded by defective genomes with important deletions. Residues 4 to 156 of the methyltransferase domain of clone C6L_[15/16] were missing, affecting the hydrophobic pocket. In addition, two of these residues (K61 and D146) are implicated in the catalytic tetrad. The hydrophobic pocket is formed by four amino acids (T104, L105, V132, and I147), and it interacts with the methyl donor, *S*-adenosyl-L-methionine, during guanosine methylation in the cap-formation process. The tetrad formed by K61, D146, K181, and E217 is implicated in catalysis [39]. Therefore, the deletion of amino acids 4-156 strongly suggests that clone C6L_[15/16] is methyltransferase incompetent.

Clone C6L_[19/20] contained a larger deletion of 228 residues in the RdRP domain, affecting several regions that have been implicated in the proper function of this domain, such as the GTP-binding domain (S710, R729, R737), which is involved in *de novo* initiation of RNA synthesis. Other regions affected by the deletion include the zinc-binding pockets (E438, H442, K446, C450, H712, H714, C728, and C847), which are implicated in conformational protein changes during RNA synthesis, and the NTP catalytic domain (GDD) [42, 43]. Clone C6L_[19/20] is therefore polymerase incompetent.

Here, the two deleted forms of the NS5 protein were detected by western blot using a polyclonal anti-NS5 antibody. The predicted molecular weight of clone C6L_[15/16] was approximately 86.2 kDa; a faint band was observed only in the protein extracts from persistently infected C6/36 cells, just below the expected molecular weight of the wild-type protein at approximately 103 kDa. In a similar manner, a band of approximately 77.4 kDa, the expected molecular weight of clone C6L_[19/20], could be detected only in the protein extracts from the persistently infected cells.

Together, our results indicate that defective DENV genomes are generated in persistently infected C6/36 cells and that some truncated proteins are translated that are likely not functional. However, the virus always preserves a wild-type version of its genome to maintain the infection. The fact that wild-type NS5 is present and expressed in persistently infected cells strongly suggests that it is functional and able to replicate both normal and defective viral genomes.

It has been reported that defective viral genomes contain regions required to recognise the viral and cellular machinery and to compete with the parental virus for this machinery; therefore, they have been implicated in controlling wild-type viral replication and in maintaining the infection [6, 7]. Recently, DENV-1 defective genomes were detected circulating in both mosquito and human populations in Yangon during an 18-month period. Their presence has been partially implicated in the reduced prevalence of DENV in that geographic area, suggesting that the defective viral genomes might regulate transmission in nature [8]. Additionally, a more recent study suggests that the presence of defective viral genomes might be involved in a reduction of the severity of dengue disease in humans [9]. However, more studies should be performed to clarify this point.

We hypothesize that the presence of defective viral genomes in our persistently infected cells is responsible for the low virus yields observed, as has been reported in other systems [4, 6, 7], but we need to perform more experiments to confirm this assumption. It is also evident that defective viral genomes are present in amounts that seem to be lower compared with the wild-type viral genomes (Fig. 9) as reported before [9], suggesting that the defective viral genomes are not preferentially replicated and making the mechanism of competence unlikely or not completely explained only by the presence of the defective genomes. This effect was also observed in the western blot assay, where the anti-NS5 antibody preferentially detected the wild-type protein over the truncated versions (Fig. 8b).

Since the virus particles released from persistently infected cells after several replicative cycles are able to infect C6/36 cells in a normal manner, it can be suggested that the mechanism of persistence might reside at the intracellular level. Several cellular mechanisms have been identified in the control of pathogen infections in mosquitoes, such as RNA interference (RNAi), Toll, and immune deficiency (Imd) pathways [48, 49]. RNAi is one of the most important innate antiviral pathways in insects. It participates in the mosquito's antiviral response against alphaviruses [50–52] and flaviviruses [53–55], and it has been suggested that it is involved in the establishment of a persistent viral infection [55] . It is triggered by the presence of dsRNAs, and previous studies have shown that when Dicer 2 (Dcr2), double-strand RNA-binding protein R2D2, and Argonaute 2 (Ago2) proteins, all of which are important components of the RNAi pathway, were silenced in *Ae aegypti* mosquitoes following infection with DENV 2, an increase in the amount of viral genomic RNA as well as a reduction in the extrinsic incubation period was observed, indicating the relevance of the small interfering RNAs (siRNAs) in the control of DENV infection in its vector [53]. However, it has been reported that the RNAi

response of C6/36 cells during viral infections is dysfunctional, mainly due to the fact that the activity of the multi-domain RNAse III Dcr2 is inefficient [56, 57] because of the presence of a homozygous frameshift mutation that creates a premature termination codon eliminating the RNAse III domains [58]. This defect has been suggested to be responsible for the high susceptibility of this cell line to flavivirus infections [56]. However, recent studies have clearly revealed that C6/36 cells possess a Dcr2-independent RNAi pathway, known as piRNA, that is activated during Sindbis virus (SINV) [59] and Chikungunya virus (CHIKV) infections [58] in this cell line. This RNAi mechanism has been identified, in addition to the classical siRNA, in the antiviral response in midguts of *Ae. Aegypti* mosquitoes infected with DENV 2 [55]. The persistently infected cells here do not display CPE, and the virus titres are low enough to be detected by conventional plaque assay, indicating a low viral replicative rate. This finding suggests that the cells are partially able to contain the viral infection, and since the canonical siRNA pathway is defective in this particular cell line, the piRNA pathway might also be responsible for this effect. Further studies are necessary to determine the participation of the piRNA pathway in the persistence process in C6/36 cells. Additionally, it would be interesting establish a persistent DENV infection in other *Aedes* mosquito cell lines such as Aag2 [60] and CCL-125 [61], where the classical Dcr-siRNA pathway is functional [58], and compare the generation of defective viral genomes and the characteristics of persistently infected cell cultures with the results observed here.

On the other hand, even though the persistently infected cells did not seem to be infected, we were always able to detect the viral genome by RT-PCR, suggesting that the antiviral response is not fully effective in eliminating the virus. The fact that DENV 2 genomic RNA accumulates in *Ae. aegypti* mosquitoes and cultured cells concurrently with increasing levels of viRNAs, strongly suggests that the virus employs some mechanism to circumvent the RNAi response [53, 55]. Experiments performed with CHIKV expressing either B2 protein from flock house virus (FHV) or Nodamura virus (NoV) have shown a significant decrease in the production of siRNAs in *Ae. albopictus* mosquitoes [58], but the mechanisms employed by arboviruses should be investigated in more detail.

There is strong evidence that the activation of the Toll pathway has an important role in the control of DENV infection in *Ae. Aegypti* mosquitoes [62, 63], but the participation of the Imd pathway seems to be more secondary, since the silencing of Caspar, an important inhibition factor, does not affect the DENV infection [62]. Unfortunately, the available information concerning the participation of these pathways during DENV infections in C6/36 cells is limited, and more studies are needed to determine both their functionality and participation during persistent infections in this cell line.

Acknowledgments We thank Dr. R. Padmanaban (Georgetown University, Washington, USA) for the anti-NS5 antibodies; Alejandro Monsalvo Reyes (FES Iztacala-UNAM), Dr. Laura Ongay, and B. Sc. Guadalupe Codiz (Instituto de Fisiología Celular-UNAM) for the DNA sequencing; and Dr. José Tapia and Nicolás Villegas (CINVESTAV-IPN) for their assistance in the northern blot hybridization. This project was supported by the Secretaría de Investigación y Posgrado of Instituto Politécnico Nacional (SIP 20010787, 20080104, 20090158, and 20100041) and Consejo Nacional de Ciencia y Tecnología (CONACYT) (Projects I39304-N and P160883). Ariadna Berenice Juárez received a scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACYT) and PIFI (IPN). Tania Olivia Vega-Almeida received a scholarship from the Instituto Politécnico Nacional and Instituto de Ciencia y Tecnología D.F. Dr. Juan Salas and Mónica De Nova have fellowships from the COFAA and EDI (IPN).

Conflict of interest The authors declare that they have no conflict of interest.

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