

# Translation efficiency determines differences in cellular infection among dengue virus type 2 strains

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## Abstract

We have investigated the molecular basis for differences in the ability of natural variants of dengue virus type 2 (DEN2) to replicate in primary human cells. The rates of virus binding, virus entry, input strand translation, and RNA stability of low-passage Thai and Nicaraguan and prototype DEN2 strains were compared. All strains exhibited equivalent binding, entry, and uncoating, and displayed comparable stability of positive strand viral RNA over time in primary cells. However, the low-passage Nicaraguan isolates were much less efficient in their ability to translate viral proteins. Sequence analysis of the full-length low-passage Nicaraguan and Thai viral genomes identified specific differences in the 3' untranslated region (3'UTR). Substitution of the different sequences into chimeric RNA reporter constructs demonstrated that the changes in the 3'UTR directly affected the efficiency of viral translation. Thus, differences in infectivity among closely related DEN2 strains correlate with efficiency of translation of input viral RNA.

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**Keywords:** Flavivirus; Dengue virus; Translation; Low-passage strains; 3' Untranslated region

## Introduction

Dengue fever (DF) is the most prevalent mosquito-borne viral disease of humans worldwide and a major public health problem in tropical and subtropical regions. It is caused by four serotypes of dengue virus (DEN), a member of the *Flaviviridae* family. DEN causes a spectrum of disease in humans, from the acute febrile illness DF to the life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Tens of millions of cases of DF occur per year, and approximately 250,000 people develop DHF/DSS annually (Burke and Monath, 2001). Despite its potential for global morbidity and mortality, the molecular

virology and pathogenesis of DEN infection are not well understood.

DEN is an enveloped, positive-sense RNA flavivirus. Its 10.7-kb genome encodes three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. Flavivirus infection begins after the virus binds to an uncharacterized cell-surface receptor and is endocytosed. After acidification induces a conformational change in the envelope protein, membrane fusion occurs and the viral nucleocapsid exits the endosome (Heinz et al., 1994). In the cytoplasm, the virus uncoats, and input positive-strand viral RNA is translated into a single polyprotein, which is then cleaved to form the structural and nonstructural viral proteins. When translation is completed, RNA replication begins with the synthesis of negative- and then positive-strand genome-length RNA, which may be used for translation of new viral proteins, synthesis of more negative-strand RNA, or encapsidation into virions. It is believed that the 5' and 3' untranslated regions (UTRs), as

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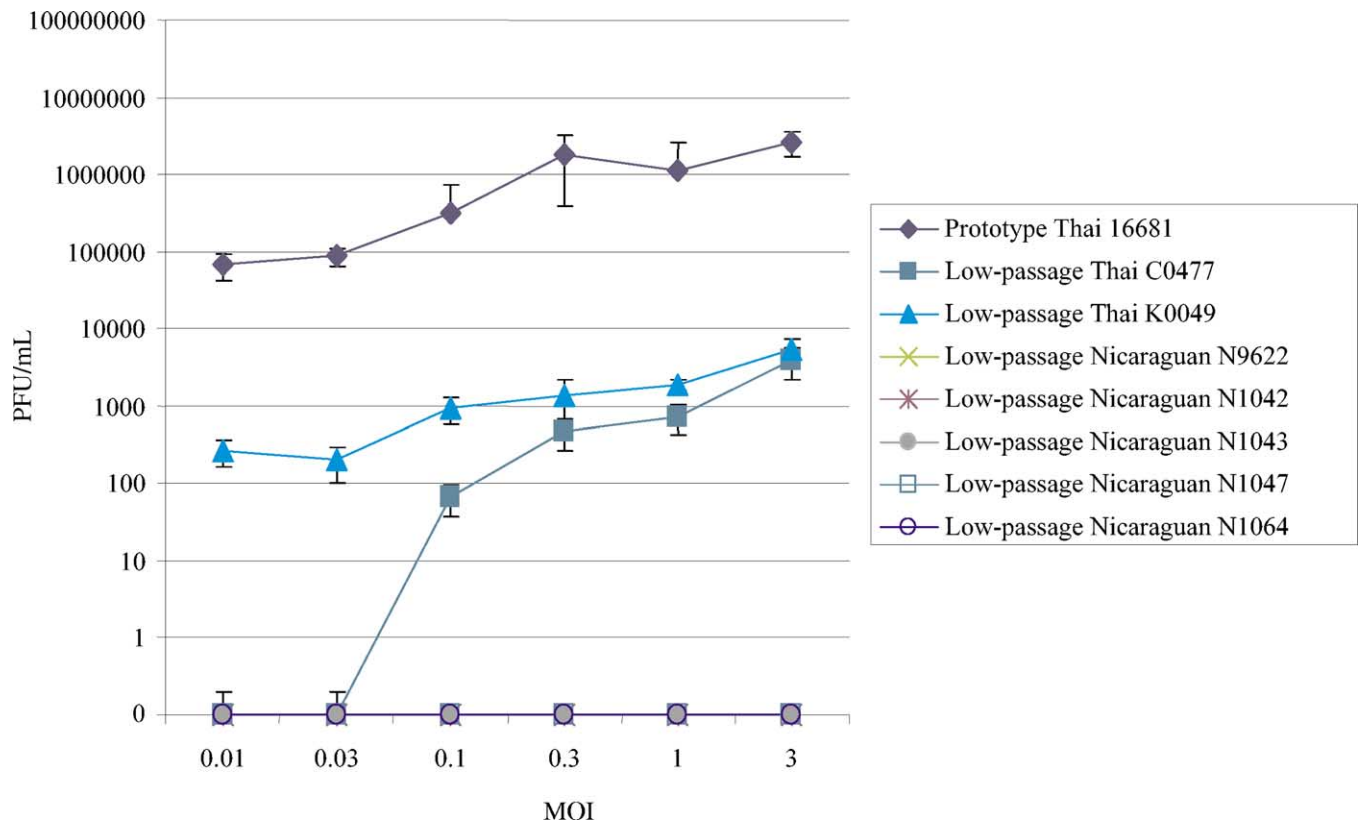


Fig. 1. Infection of HFF by different strains of DEN2. HFF cells were exposed to Thai DEN2 strains (16681, C0477, and K0049) or Nicaraguan DEN2 isolates (N1042, N1043, N1047, N1064, and N9622) over a range of MOIs and incubated for 72 h. Supernatants were harvested for plaque assays. The plaque assay data are expressed as numbers of PFU per milliliter. The data are the averages of three experiments, and the error bars represent the standard deviation.

well as the NS1, NS2A, NS3, NS4A, and NS5 proteins, are involved in the formation of a replication complex (Khromykh et al., 2000; You et al., 2001). Finally, the virus uses the host secretory system to escape the cell.

Previously, we evaluated cellular infection with low-passage viral isolates by asymmetric semiquantitative RT-PCR, flow cytometry, and plaque assays that measured viral RNA levels, viral antigen expression, and virion production, respectively. In human foreskin fibroblasts (HFF) and myeloid cells, striking differences were observed in the ability of different strains of DEN2 to productively infect cells. The prototype Thai DEN2 strain (16681) was able to infect HFF and myeloid cells, as were isolates of the low-passage Thai strain, albeit less efficiently. In contrast, isolates of the low-passage Nicaraguan strain were unable to productively infect HFF and myeloid cells, as determined by lack of accumulation of negative-strand viral RNA, intracellular antigen, and infectious viral progeny (Diamond et al., 2000a). Because these closely related DEN2 strains exhibited distinct phenotypes within the same cells, we hypothesized that specific genetic determinants define functional differences in particular steps in the viral life cycle. In this report, we show that the efficiency of translation of input viral RNA may be a major determinant of the observed differences in infectivity among DEN2 strains.

## Results

We previously had found functional differences between closely related low-passage DEN2 isolates and a highly passaged prototype DEN2 strain in their ability to productively infect human cells (Diamond et al., 2000a). Data from these experiments suggested that low-passage DEN2 isolates were less efficient than the prototype strain at productively infecting primary human myeloid and fibroblast cells and that differences existed between low-passage strains of distinct geographic origin. Specifically, the prototype Thai and isolates of the low-passage Thai DEN2 strain were able to productively infect HFF and myeloid cells, whereas isolates of the low-passage Nicaraguan strain were not. We chose to continue our studies in HFF cells because they yielded less donor-to-donor variability than myeloid cells. As no discernible difference in function was detected among isolates from each of the strains in HFF cells (Fig. 1), multiple isolates of both the Thai (K0049 and C0477) and Nicaraguan (N9622, N1042, N1043, N1047, and N1064) strains were used in each of the experiments described below. Because these closely related DEN2 strains exhibited distinct phenotypes within the same cells, we hypothesized that specific genetic determinants defined functional differences in particular steps in the viral life cycle.

To confirm that the decreased efficiency of the Nicara-

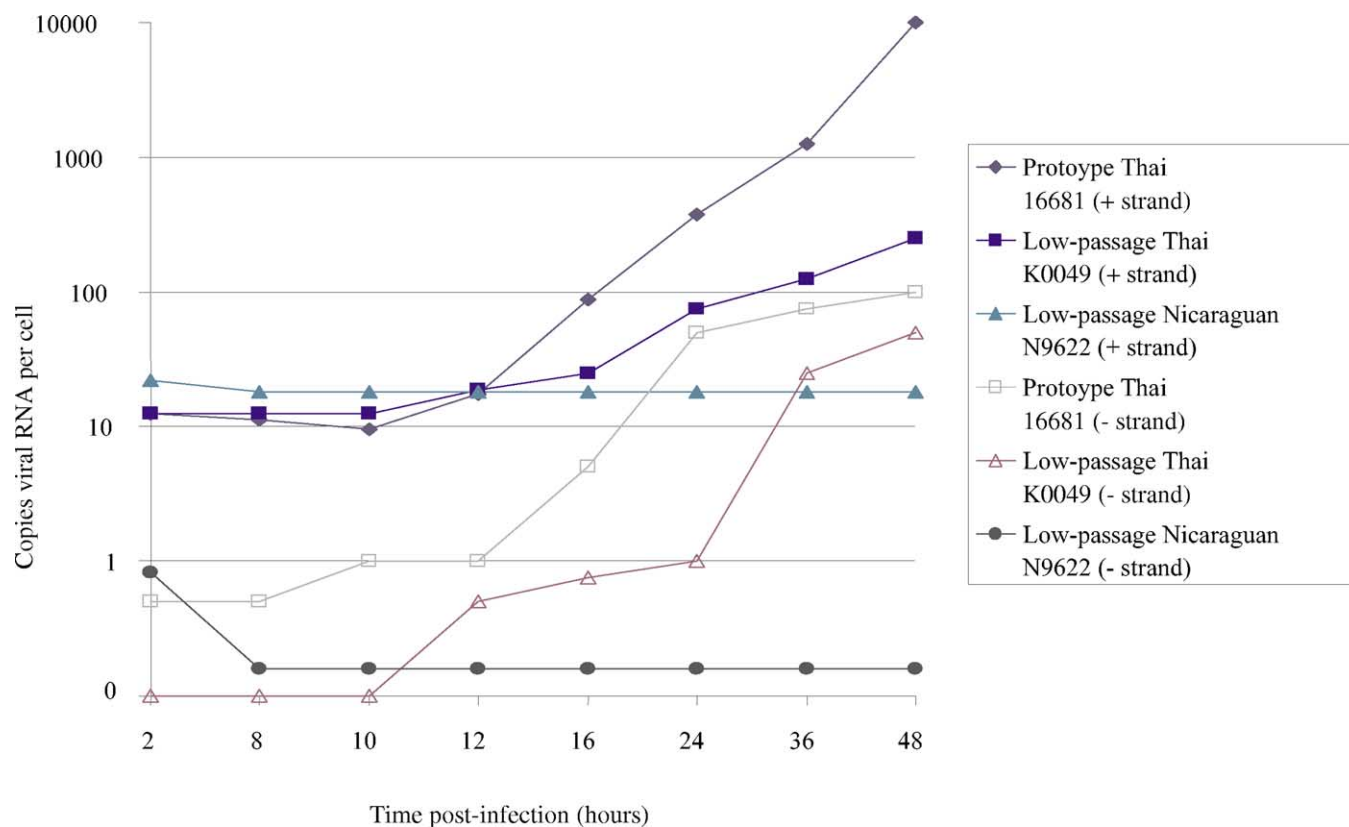


Fig. 2. Determination of DEN2 positive- and negative-strand RNA stability. Cells were exposed to Thai strains (16681, C0477, and K0049) or Nicaraguan isolates (N9622 and N1043) at an MOI of 1 and harvested at specific time points post infection. Samples taken at the 2-h time point were first washed with 1 M NaCl, pH 9.5, to remove surface-bound viral particles. Total RNA was isolated and subjected to asymmetric, semiquantitative competitive RT-PCR using an antisense primer or a sense primer for reverse transcription to measure the positive-strand RNA or the negative-strand RNA, respectively. The data are expressed as number of copies of viral RNA per cell and are representative of three experiments.

guan strain to productively infect HFF cells was not due to the absence of intracellular viral RNA, the levels of positive- and negative-strand viral RNA were measured in infected HFF cells over time by asymmetric semiquantitative RT-PCR. To ensure that the viral particles being measured were in fact intracellular and not simply bound to the cell surface, an alkaline high-salt solution (pH 9.5, 1 M NaCl) was used to remove surface-bound virus at the 2-h time point post infection; this treatment removes ~95% of viral RNA associated with cells at 4°C (Diamond and Harris, 2001). While both the prototype and low-passage Thai DEN2 strains exhibited an increase in levels of positive-strand and negative-strand viral RNA over time, reflecting viral replication, no increase in the levels of positive- or negative-strand viral RNA was detected in cells infected with the low-passage Nicaraguan strain from entry until at least 48 h after infection (Fig. 2). However, intracellular positive-strand viral RNA from the Nicaraguan strain was detected at all time points tested. These constant levels of intracellular Nicaraguan DEN2 viral RNA over time may be attributable to either stability of the viral genome or low-level replication resulting in persistence of positive-strand viral RNA within the cell. In either case, these results indicate that the inability of the Nicaraguan strain to pro-

ductively infect HFF cells is not due to the lack of viral RNA in the cell.

#### *The DEN2 strains do not differ significantly at early stages of infection*

To further characterize the functional differences between the DEN2 strains, assays were developed to examine the efficiency of the viral strains in proceeding through the early stages of infection. Binding was measured by quantitating the viral RNA associated with the cell surface after incubation with DEN2 strains at 4°C, which prevents internalization of the virus (Bielefeldt-Ohmann, 1998). Quantitation of viral RNA was performed using a real-time RT-PCR assay. We have previously shown that measuring viral adsorption by detecting viral RNA is equivalent to detection of radiolabeled or immunofluorescent surface-bound particles (Diamond and Harris, 2001) (data not shown). The ability of the distinct viral strains to bind to the cells was not markedly different; on average, an approximately two-fold difference in binding between the Thai and Nicaraguan strains was observed (Fig. 3, “4°C no salt”). To confirm that viral particles bound to the cell surface were being measured, an alkaline high-salt solution (pH 9.5, 1 M NaCl) was

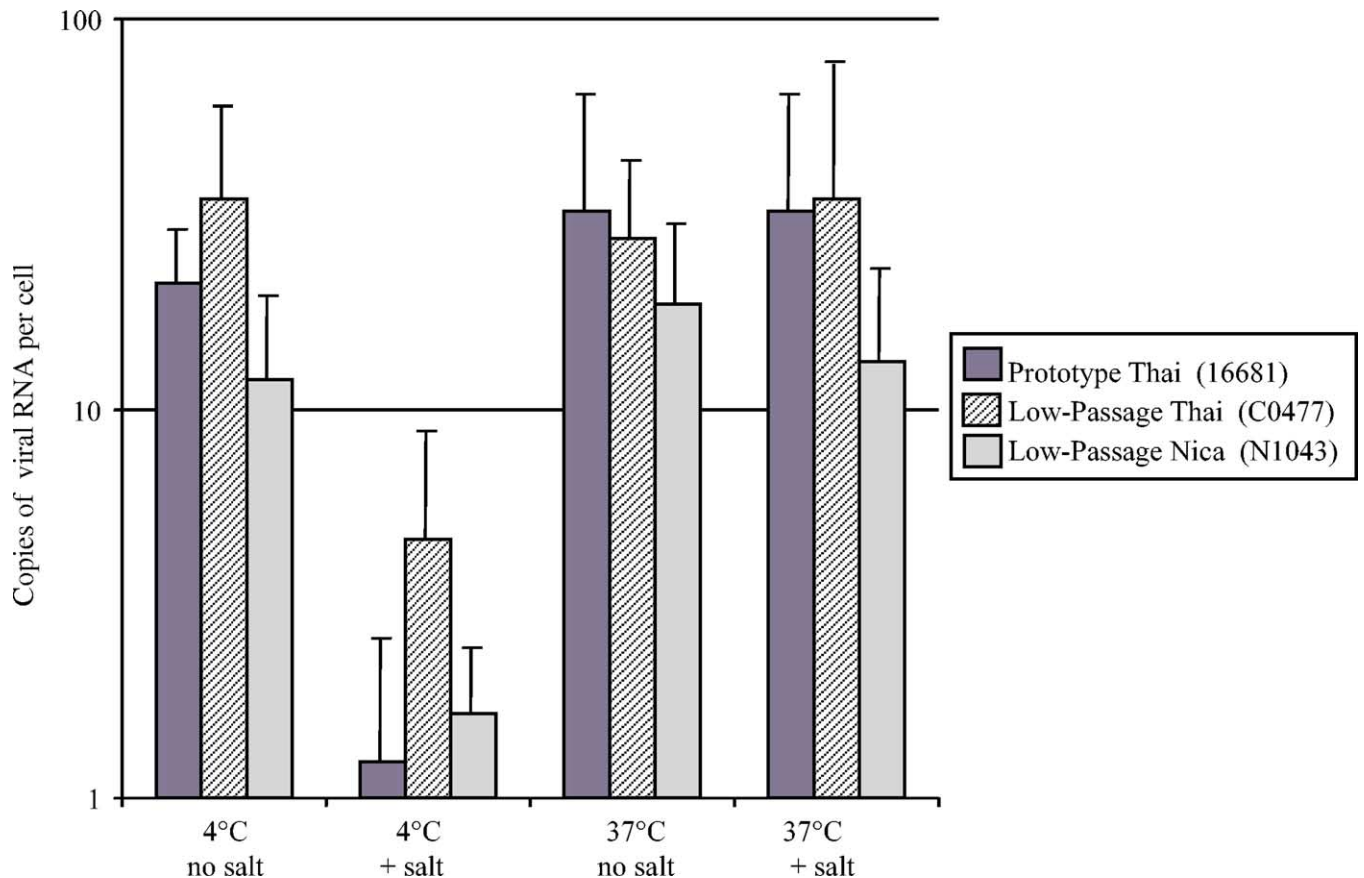


Fig. 3. DEN2 binding and entry into HFF. HFF were incubated with 16681, low-passage Thai (C0477), or Nicaraguan (N1064, N1047, and N1043) DEN2 strains at an MOI of 1 at 4°C or 37°C for 2 h as indicated. Duplicate samples were treated with an alkaline, high-salt solution (“+salt”) for 2 min to remove surface-bound virus. After extensive washing, total RNA was extracted from harvested cells, and positive-strand RNA was measured by real-time RT-PCR. The data are expressed as the average of three experiments, and the error bars represent the standard deviation. Similar results were obtained with Nicaraguan isolates N1064 and N1047.

used to remove surface-bound virus, as above (Diamond and Harris, 2001); this treatment removed 75–95% of viral RNA associated with cells at 4°C (Fig. 3, “4°C + salt”). Viral entry was measured by detection of viral RNA in the cytoplasm after exposure of the cells to DEN2 strains at 37°C, at which temperature virions are internalized (Fig. 3, “37°C no salt”). Again, an alkaline high-salt wash was used to remove surface-bound virus, ensuring that any remaining virus was inside the cell (Diamond and Harris, 2001). Again, no significant difference in the abilities of the prototype Thai, low-passage Thai, and low-passage Nicaraguan isolates to enter cells at equivalent multiplicity of infection (MOI) (Fig. 3, “37°C + salt”).

We next investigated whether the difference in infection was due to a differential ability of each viral strain to uncoat efficiently in the cytoplasm. Because we were unable to directly measure nucleocapsid uncoating of the virus, we opted to indirectly measure the ability of the virus to complete the early stages of infection after entry. To do this, the natural route of infection was bypassed by transfecting equivalent amounts of infectious positive-strand viral RNA directly into the cytoplasm of HFF and the control cell line

BHK. If the difference between the viral strains were due to a step in the viral life cycle downstream of uncoating, then the differences in infectivity between the viral strains should remain. However, if the inefficiency of viral infectivity was due to a step upstream of viral uncoating, then the transfected viral RNAs should infect the cells equivalently. Productive infection was measured by plaque assay of cellular supernatant 48 h after transfection. The results of this experiment demonstrate that although all the DEN2 strains were capable of growth in BHK cells after transfection, the DEN2 prototype and primary strain RNAs continued to exhibit the same differences in productive infection of HFF cells (Fig. 4), suggesting that the step in the viral life cycle that distinguished the infectivity of the strains in the primary cells was downstream of viral uncoating. Moreover, the amount of positive-strand viral RNA detected inside cells at 4 h was equivalent to that detected at 24 h after transfection with the low-passage Nicaraguan DEN2 strain. Since positive-strand viral RNA was stable over time and no negative-strand RNA was detected in cells transfected with the low-passage Nicaraguan DEN2 strain (data not shown)—thus precluding synthesis of additional positive-strand viral

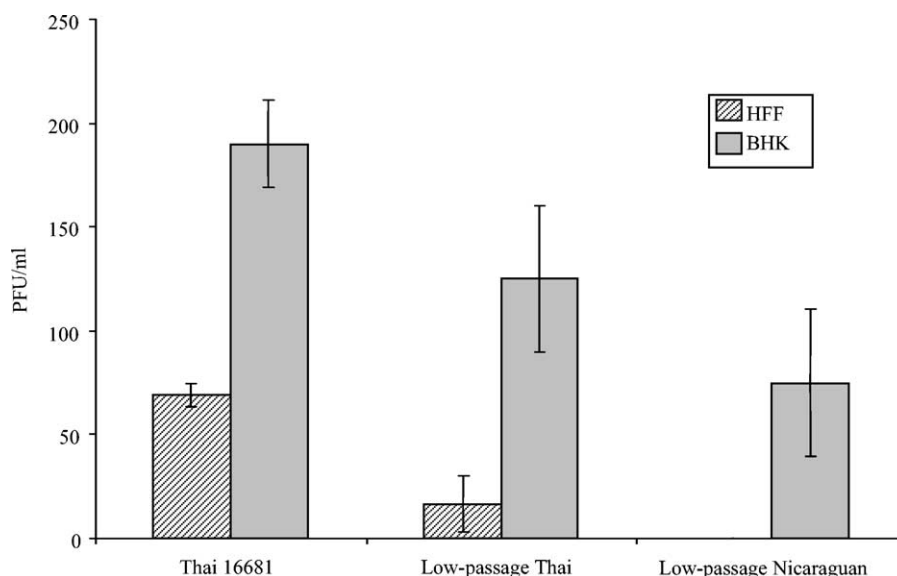


Fig. 4. Transfection of viral RNA. Viral RNA from prototype Thai (16681), low-passage Thai (C0477 and K0049), or Nicaraguan DEN2 (N9622, N1047, and N1043) isolates was harvested from the supernatant of infected C6/36 cells, and viral RNA was extracted. HFF and BHK-21 cells were transfected with equal amounts of positive-strand viral RNA in the presence of DMRIE-C liposomal reagent and transfection medium. After 4 h, monolayers were washed and incubated for 48 h at 37°C. Levels of infectious virus were assessed by plaque assays using BHK-21 cells. The data are expressed as an average of three experiments, and the error bars represent the standard deviation.

RNA—we conclude that the transfected Nicaraguan strain positive-strand viral RNA is not being degraded at an increased rate. Therefore, the distinct replication phenotype of the Nicaraguan strain in primary cells appeared not to be due to a differential stability of the viral RNA after transfection but rather to a step in the viral life cycle downstream of viral uncoating.

*The low-passage Nicaraguan DEN2 strain is translated less efficiently than the DEN2 Thai strains*

The relative translation efficiency of each strain was next investigated. Several approaches were attempted to measure input strand translation of low-passage Thai and Nicaraguan DEN2 strains in HFF cells at early time points post infection. Initially, the ability of each strain to translate viral RNA was investigated by immunoprecipitation of metabolically labeled viral proteins produced in HFF. Cells were infected with each of the DEN2 strains and pulsed with  $^{35}\text{S}$ -cysteine-methionine for 30 min at 14 and 24 h post infection. The cells were then lysed, and the E protein was immunoprecipitated (Fig. 5A). Labeled E protein was detected at 24 h post infection in HFF cells infected with 16681 or low-passage Thai strains but not with the Nicaraguan strain, suggesting that translation of the Nicaraguan strain is less efficient. However, despite the fact that the low-passage Thai strain productively infected HFF cells, using this method, we were unable to detect translation of either the Thai or Nicaraguan low-passage strains at earlier time points and thus required a different approach to examine translation of input strand viral RNA. Therefore, the

relative translation efficiencies of the strains were assayed by metabolic labeling in BHK cells that translate viral RNAs more efficiently, thus allowing a lower limit of detection than HFF cells. To detect the minute quantities of protein translated from input strand viral RNA, cells were infected with each of the DEN2 strains and exposed to a high-salt solution that decreases translation of cellular proteins more than that of viral proteins (Erdei, 1981; Schrader and Westaway, 1990). We have shown previously that synthesis of both 16681 DEN2 negative- and positive-strand RNA in infected BHK cells begins 6–8 h post infection, whereas no increase in the levels of either strand is found at 4 h post infection (Diamond et al., 2002). Therefore, to measure translation of input strand viral RNA versus replicated viral RNA, cells were pulsed with  $^{35}\text{S}$ -cysteine-methionine for 30 min at 4 and 24 h post infection, respectively. Lysates were subjected to electrophoresis (Fig. 5B), and viral translation was measured by quantifying the intensity of the radiolabeled NS5 protein, the most readily observable of the viral proteins (Fig. 5C). The identity of this band as NS5 was verified by Western blot analysis (Diamond et al., 2002) (data not shown). At 4 h post infection, input strand translation of the low-passage Thai strain was ~50% of the prototype Thai strain 16681, whereas translation of the low-passage Nicaraguan strains was undetectable. These results are mirrored at 24 h post infection in BHK cells, with a 5-fold or a 10-fold difference in measurable translation between the prototype Thai strain 16681 and the low-passage Thai or the low-passage Nicaraguan strain, respectively. These results suggest that translation of input viral RNA is a rate-limiting step that ac-

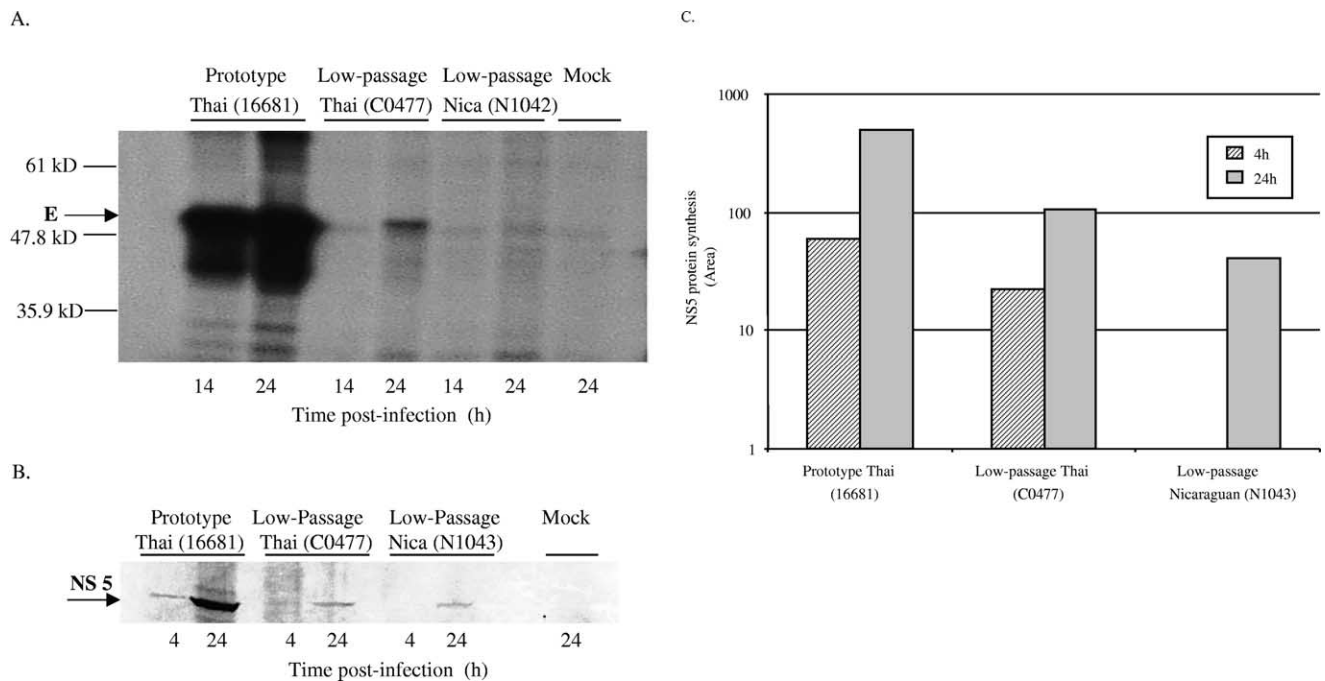


Fig. 5. Translation of DEN2 RNA. (A) Immunoprecipitation of DEN2 E protein in HFF cells. HFF were infected with 16681, low-passage Thai (C0477), or Nicaraguan (N1047 and N1043) strains of DEN2 at an MOI of 100 and incubated until the specified time points. Immunoprecipitations were performed with anti-DEN2 E mAb and analyzed by SDS-PAGE as detailed in the materials and methods section. (B) Translation of DEN2 NS5 in BHK cells. BHK cells were infected with 16681, low-passage Thai (C0477), or Nicaraguan DEN2 (N1047) strains of DEN2 and incubated until the specified time points. Cells were labeled with  $^{35}\text{S}$ -cysteine-methionine, and lysates were analyzed by SDS-PAGE. Labeled proteins were then visualized using the PhosphorImager detection system. (C) Quantitation of DEN2 translation in BHK cells. Translation of DEN NS5 protein was quantified by measuring the area under a peak generated from a density profile of each protein band. The data are representative of two experiments.

counts, at least in part, for the disparate abilities of DEN2 strains to infect both cell types.

This inefficiency of translation of the Nicaraguan strains appeared to be more pronounced in primary HFF than in the BHK cell line, and in these primary HFF cells could lead to exceedingly low levels of the nonstructural proteins that are necessary for replication of the viral RNA genome. Thus, it would be expected that there is no *de novo* synthesis of viral RNA in HFF cells infected with the Nicaraguan strain. In cells infected with the different strains, nascent RNA was biosynthetically labeled with  $^{32}\text{P}_i$ . As predicted, viral RNA synthesis occurred in HFF cells infected with the prototype Thai DEN2 but not with low-passage Nicaraguan strain (Fig. 6A). The 11-kb band was confirmed to be DEN2 viral RNA by Northern analysis (Fig. 6B). This result is consistent with the initial observation that HFF cells infected with the Nicaraguan isolates did not accumulate negative-strand viral RNA (Diamond et al., 2000a).

#### *Conserved sequence differences exist between the low-passage Thai and Nicaraguan DEN2 strains*

The observed difference in the efficiency of translation among the strains examined is likely due to a combination of cellular and viral factors. To examine potential viral determinants, a representative low-passage Thai (C0477)

and a low-passage Nicaraguan strain (N1042) were sequenced in their entirety. The strains are closely related, demonstrating 98.1% identity (data not shown). To identify the viral sequence differences that may confer the observed distinct phenotypes, the Thai and Nicaraguan sequences were aligned with other full-length DEN2 sequences from the Asian/American-Asian genotype (see materials and methods section). Conserved amino acid or nucleotide differences were considered to be those that were common among all isolates within a strain but differed between the Thai and Nicaraguan strains. Several reproducible differences between the Nicaraguan and Thai strains were revealed. Non-neutral substitutions in prM (S15G), E (N203D), NS4B (Q22E), and NS5 (Y47H, E558K) were observed, and 10 nucleotide changes were found in the 3'UTR (Table 1). Sequence analysis of additional Nicaraguan isolates (N1047 and N1043) detected the same nucleotide differences (data not shown). These nucleotide differences could influence the translation efficiency of input viral RNA.

#### *Sequences in the 3'UTR modulate DEN translation efficiency*

Because the 3'UTR has been speculated to play a role in viral translation (Dreher, 1999; Guo et al., 2001; Ito et al.,

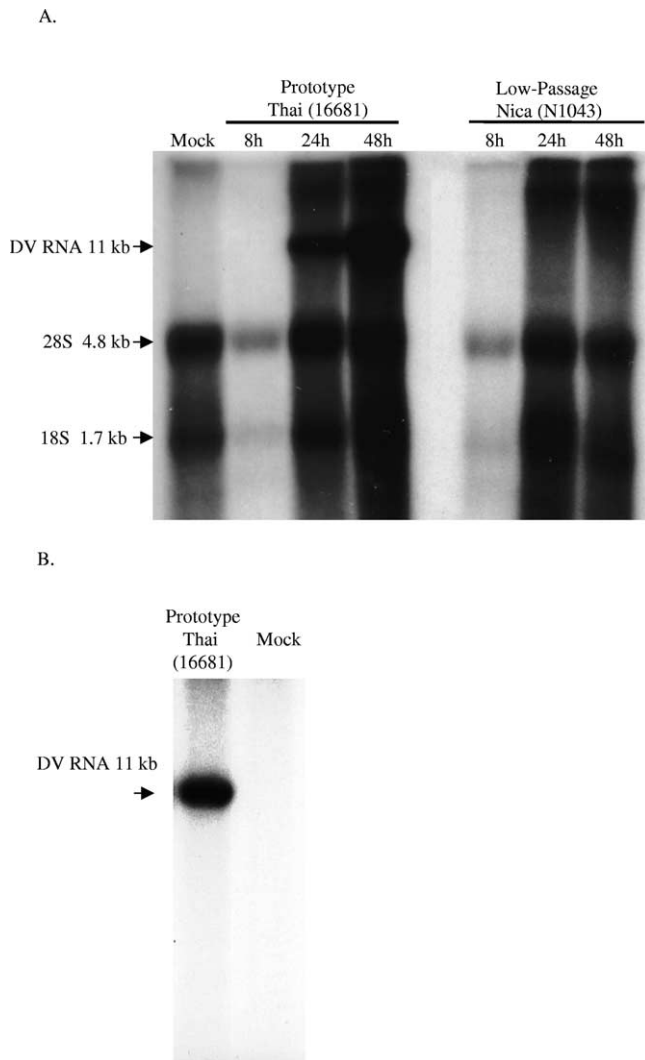


Fig. 6. Synthesis of DEN2 RNA. (A) Biosynthetic labeling of DEN2 RNA. HFF were infected with 16681 or Nicaraguan DEN2 (N9622, N1047, and N1043) at an MOI of 5 for 2 h, washed with serum-free medium, and treated with actinomycin D in DMEM containing 5% phosphate for 1 h to inhibit cellular RNA synthesis; 300  $\mu$ Ci of  $^{32}$ P<sub>i</sub> was added to each well, and the cells were harvested at the specified time points post infection. Total cellular RNA was extracted, and the RNA was visualized by formaldehyde gel electrophoresis and autoradiography. (B) Northern blot detecting DEN2 RNA. HFF cells were infected, as above. At 24 h post infection, cells were harvested, and the identity of the 11-kb band of RNA was confirmed as DEN2 by Northern blot analysis of total RNA separated by formaldehyde gel electrophoresis. A DIG-labeled RNA probe directed against a 453-nt fragment of the DEN2 3'UTR was hybridized to membrane-crosslinked samples, which were detected by chemiluminescent substrate and visualized by exposure to radiographic film.

1998; Piron et al., 1998), we decided to analyze the effect of the sequence differences in the DEN2 3'UTRs on translation efficiency. The distinct 3'UTRs were amplified and cloned into a chimeric RNA reporter system (Holden, K., Harris, E., unpublished data). This reporter construct consists of the 5'UTR of DEN2 (this sequence is identical among the Thai and Nicaraguan strains) followed by the first 72 nucleotides of the prototype Thai (16681) capsid

coding sequence fused to the firefly luciferase gene, and, finally, the 3'UTR of either 16681, the low-passage Thai (C0477), or the low-passage Nicaraguan strains (N1042) (Fig. 7A). These constructs were then tested for luciferase activity in an in vitro translation system generated from BHK or HFF cells, and RNA quantity and stability were monitored in each experiment. Quantitation of RNA by Northern blot indicated that the amount and stability of the three RNA reporter constructs in the in vitro translation extracts were comparable (Fig. 7B).

Though the efficiency of translation of each extract varied among different preparations, the constructs exhibited the same pattern of translation efficiency regardless of the extract preparation (Table 2). In both BHK and HFF in vitro extracts, translation of each of the 3'UTR constructs paralleled the replication phenotype of the different strains. Specifically, in the BHK extracts, translation of the chimera bearing the low-passage Thai 3'UTR (5DENLuc3DEN-Thai) was reduced by 33%, and translation of the construct containing the Nicaraguan 3'UTR (5DENLuc3DEN-Nica) construct was reduced by 65% in comparison to the construct containing the prototype Thai 3'UTR (Fig. 7C). The disparity between the strains was even more striking in the HFF cells in vitro translation system. Here, when compared to the prototype 5DENLuc3DEN-16681 construct, translation of the 5DENLuc3DEN-Thai construct was reduced by 42%, and translation of the 5DENLuc3DEN-Nica construct was reduced by 82% (Fig. 7D). Thus, the chimera with the Nicaraguan 3'UTR was 3-fold less efficient at translation in BHK extracts and 5-fold less efficient in HFF extracts than the chimera containing the prototype Thai 16681 3'UTR. The percent difference in luciferase activity of the 5DENLuc3DEN-Nica construct compared to either the 5DENLuc3DEN-16681 or the 5DENLuc3DEN-Thai constructs was statistically significant ( $P < 0.01$ , Student's *t* test) in both BHK and HFF extracts. Quantitation of RNA by Northern blot analysis and normalization of relative luciferase units by RNA quantity yielded a similar trend over a range of concentrations of input RNA (Fig. 7B, and Table 2, 4A and 4B). These results demonstrate that sequences within the 3'UTR are directly involved in translation of the viral genome and may be at least partially responsible for the lack of replication of the low-passage DEN2 strains in HFF cells.

## Discussion

We have identified functional differences in the ability of DEN2 natural variants to replicate within human cell types. Previous results indicated that whereas the highly passaged prototype DEN2 strain efficiently infected HFF and myeloid cells, low-passage strains were less efficient at certain steps of the infection process (Diamond et al., 2000a). In this study, we investigated the molecular basis for differences in the ability of natural variants of DEN2 to replicate in HFF.

Table 1

Summary of consistent amino acid and nucleotide changes identified between Thai and Nicaraguan DEN2 viruses indicated according to nucleotide position within the viral genome

Gene	prM	E	NS4b	NS5	NS5	3'UTR										
Amino acid position <sup>b</sup>	Amino acid changes <sup>a</sup>					Nucleotide changes <sup>a</sup>										
	15	203	22	47	558	10299	10387	10396	10411	10449	10538	10551	10566	10571	10605	
DEN strain																
Thai 16681	S	N	Q	R	E	g	t	t	a	c	t	c	g	g	g	
Thai C0477	S	N	Q	R	E	a	t	t	a	t	t	c	g	g	g	
Nicaraguan N1042	G	D	E	K	K	a	c	c	g	t	c	t	a	a	a	

<sup>a</sup> Uppercase letters: amino acid; lowercase letter: nucleotides.

<sup>b</sup> Amino acid number denotes position within each gene.

<sup>c</sup> Position as per sequence of DEN2 strain 16681 (Accession no.: U87411).

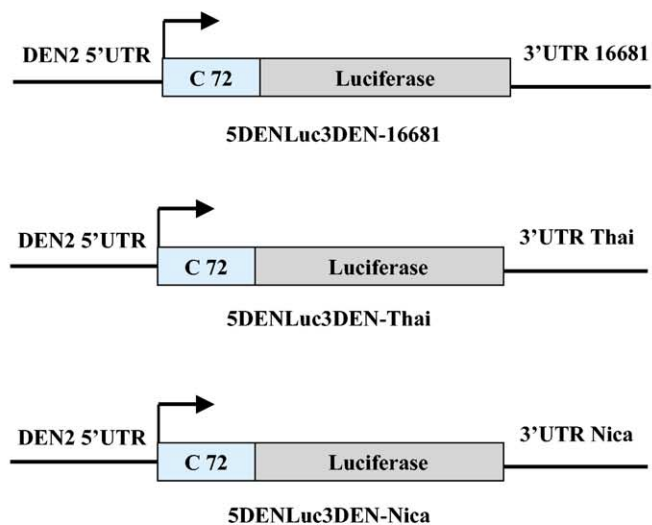
We compared the rates of virus binding, virus entry, viral RNA stability, input strand translation, and protein expression of low-passage Thai and Nicaraguan isolates and the prototype Thai DEN2 strain 16681. In all assays, multiple low-passage isolates of Thai and Nicaraguan origin were used. At equivalent MOI, all strains exhibited similar binding and entry ( $\leq 2$ -fold difference) and displayed comparable stability of positive-strand viral RNA over time in HFF cells. The DEN2 strains continued to exhibit differences in productive infection of HFF cells upon transfection of infectious viral RNA directly into the cytoplasm, suggesting that the step that distinguished the infectivity between strains was downstream of viral uncoating. We found that in contrast to the prototype and low-passage Thai strains, the low-passage Nicaraguan isolates were much less efficient in their ability to translate viral proteins from infectious viral RNA. In addition, we identified sequence differences in the 3'UTRs of the different strains and correlated these differences with decreased translation efficiency through the use of reporter constructs. Consistent with these findings, viral RNA synthesis was shown to occur in HFF cells infected with either the prototype Thai DEN2 or the primary Thai strain but not with the low-passage Nicaraguan strain (Fig. 6A and data not shown). Thus, we have exploited the distinct replication phenotypes of the prototype and low-passage strains in HFF to identify critical steps in the viral life cycle and have shown that one rate-limiting step among different DEN strains is translation of viral RNA into protein.

Translation is a tightly regulated process in the host cell (Matthews et al., 2000). It is a key point of control for many viruses, which are entirely dependent on the host cell protein synthesis machinery (Gale et al., 2000). This is particularly true for positive-strand RNA viruses, as it has been shown in both picorna- and flaviviruses that translation of

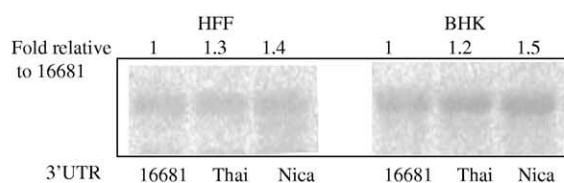
each viral RNA genome is coupled to replication (Egger et al., 2000; Khromykh et al., 1999, 2000; Novak and Kirkegaard, 1994), and that replication is coupled to assembly (Khromykh et al., 2001b; Nugent et al., 1999). As another affirmation of the key role of translation, many viruses have developed strategies to subvert the host translation machinery (Gale et al., 2000). In this report we show that a decreased efficiency in translation of the DEN2 Nicaraguan strains explains, at least in part, the reduction in the ability of the virus to productively infect primary cells.

We show that sequences in the DEN 3'UTR directly affect the translation efficiency of DEN reporter constructs. This is consistent with existing data, as stimulation of translation by the 3'UTR has been observed in both cellular and viral systems (Guo et al., 2001; Ito et al., 1998; Piron et al., 1998; Sachs, 2000; Sonoda and Wharton, 1999). In addition, our laboratory has recently demonstrated that the 3'UTR of DEN2 stimulates translation (Holden, K., Harris, E., unpublished data). Previous studies have revealed that sequence differences within the DEN 5'UTR and 3'UTR are predicted to change RNA secondary structure as well as to confer a less virulent phenotype in vitro (Butrapet et al., 2000; Leitmeyer et al., 1999). Substitution or deletion mutations in the flavivirus untranslated regions cause attenuation both in vitro and in vivo (Blaney et al., 2002; Cahour et al., 1995; Men et al., 1996). The DEN2 3'UTR is 453 nucleotides long and, like all flaviviruses, contains a highly conserved 3' stem loop (SL) and a conserved cyclization domain (CD) (Hahn et al., 1987). Perturbation of the 3'SL or the 3'CD has been shown to interfere with DEN2 RNA synthesis and viral viability (Khromykh et al., 2001a; You and Padmanabhan, 1999; Zeng et al., 1998). However, these studies did not address the effect on viral translation. Interestingly, the nucleotide differences described here are upstream of the 3'SL and lie outside the 3'CD, suggesting that

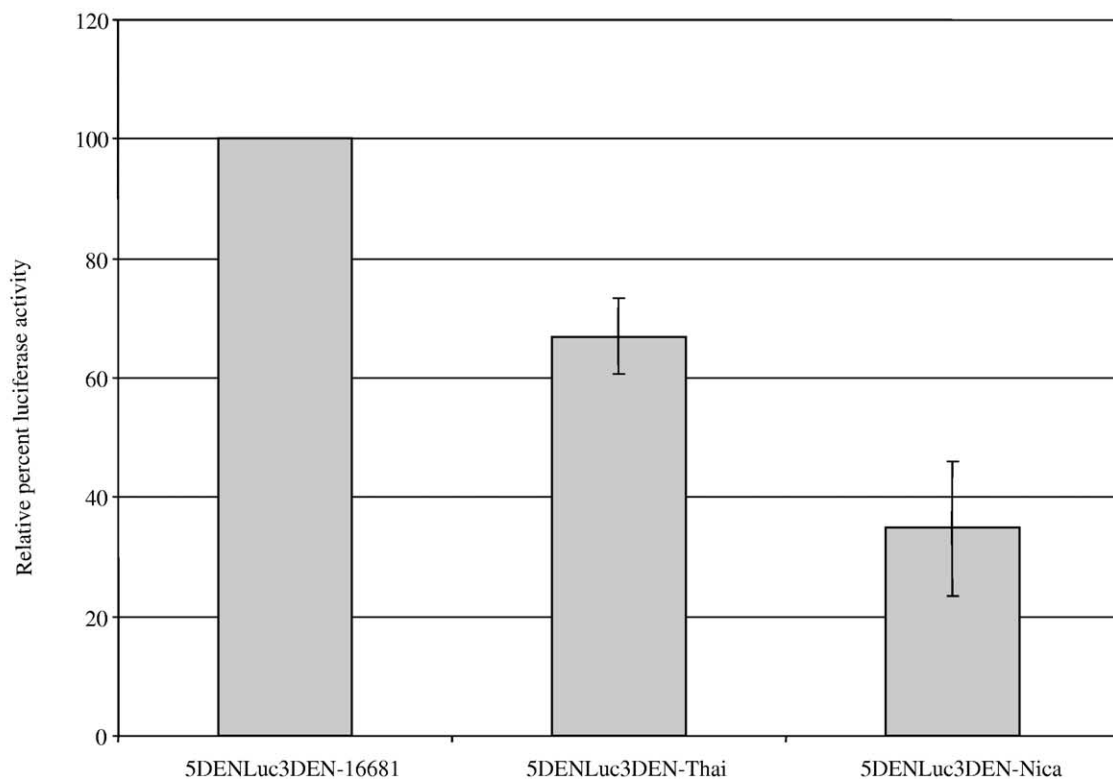
A.



B.



C.



D.

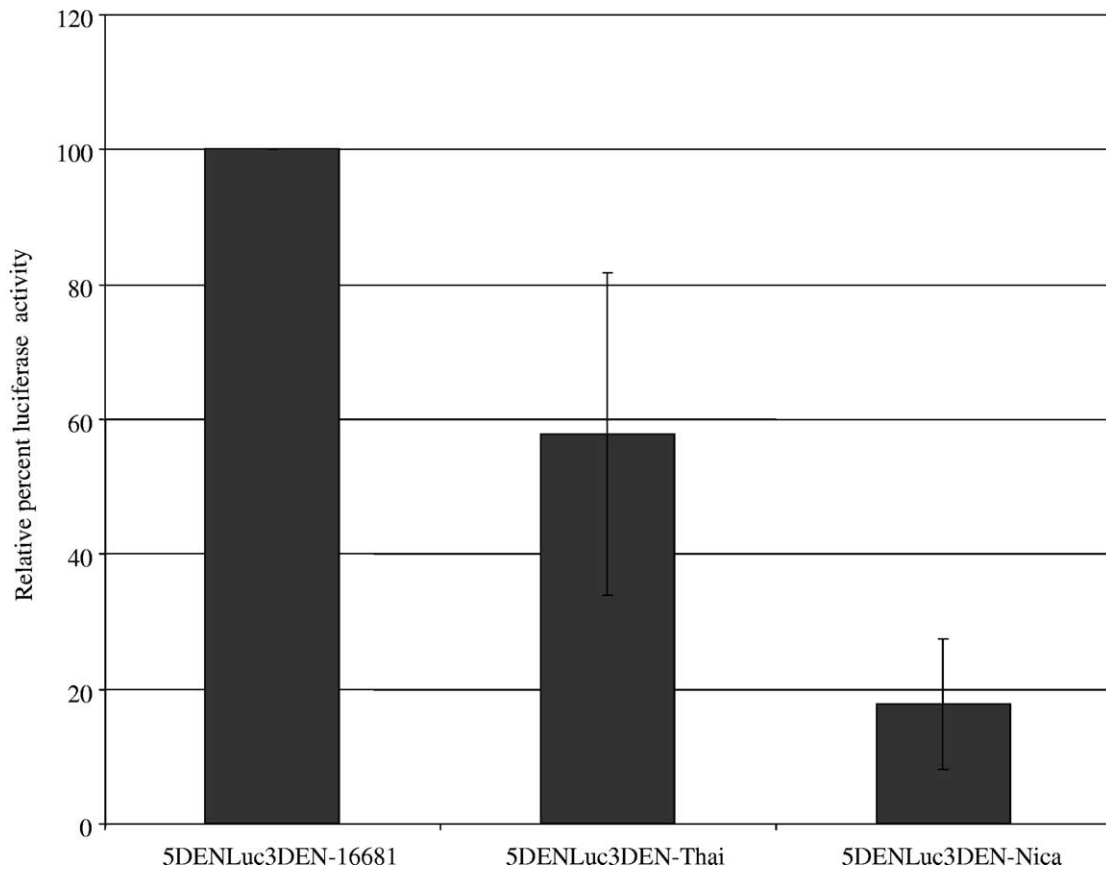


Fig. 7 (continued)

additional sequences or structures in the 3'UTR may directly mediate interactions critical for translation.

The functional significance of the sequence differences described in this study may be explained through an effect on *cis*-acting regulatory elements in the 3'UTR that disrupt key RNA-RNA or RNA-protein interactions. For instance, sequence differences between the DEN2 strains in this study may influence translation efficiency by lowering the affinity

of viral sequences for critical host translation factors. Certain initiation factors, such as eIF4G, eIF4A, and eIF3 (Pestova et al., 2001), are presumably required for cap-dependent translation of dengue virus. Noncanonical factors such as eEF1A (eIF1 $\alpha$ ) (Harris et al., 1994), PTB (Hunt and Jackson, 1999), and La antigen (De Nova-Ocampo et al., 2002; Meerovitch et al., 1993), which are crucial for the translation of several picornaviruses and have been shown

Fig. 7. Translation of DEN2 chimeric reporter constructs in BHK and HFF in vitro translation extracts. (A) Schematic diagrams of the reporter constructs used in this study. Chimeric RNA constructs consisted of the 16681 DEN2 5'UTR sequence, the first 72 nucleotides of the capsid (C) region of 16681 fused to the firefly luciferase (Luc) gene, and either the 16681, the low-passage Thai (C0477), or the low-passage Nica (N1042) DEN2 3'UTR sequence (5DENLuc3DEN-16681, 5DENLuc3DEN-Thai, or 5DENLuc3DEN-Nica, respectively). The arrow designates the translation start site. (B) Northern blot detecting DEN2 RNA reporter constructs in BHK and HFF in vitro translation extracts. The stability of RNA reporter constructs following a 60-min incubation in in vitro translation extracts was analyzed by Northern blot analysis of total RNA separated by formaldehyde gel electrophoresis and transferred to nylon membrane. A  $^{32}$ P-labeled RNA probe, which targets a 453-nt fragment of the DEN2 3'UTR, was hybridized to the RNA samples crosslinked to the membrane. 5DENLuc3DEN RNA was quantified by measuring the area under a peak generated from a density profile of each protein band. This measurement was used to normalize experiments 4A and B shown in Table 2. (C) Translation of chimeric constructs relative to 16681 in BHK in vitro translation extracts. In vitro transcripts of each construct were added to in vitro translation extracts prepared from BHK cells as described in the materials and methods section. The extracts were incubated at 30°C for 60 min, after which luciferase activity was assayed by luminometry. The data are expressed as the average of four experiments, and the error bars represent the standard deviation. (D) Translation of chimeric constructs relative to 16681 in HFF in vitro translation extracts. In vitro transcripts of each construct were added to in vitro translation extracts prepared from HFF as described in the materials and methods section and were processed as above. The data are expressed as the average of four experiments, and the error bars represent the standard deviation.

Table 2

Summary of luciferase activity obtained in BHK and HFF in vitro translation extracts from reporter constructs containing distinct DEN2 3'UTRs

Reporter Construct	Translation extract									
	BHK					HFF				
	Experiment									
	1	2	3	4A <sup>b,c</sup>	4B <sup>b,d</sup>	1	2	3	4A <sup>b,c</sup>	4B <sup>b,d</sup>
5DENLuc 3DEN-16681	10020 <sup>a</sup>	12218	11325	4114	490	565	6761	339	174	20
5DENLuc 3DEN-Thai	8919	10451	9851	2680	206	352	3365	300	54	3
5DENLuc 3DEN-Nica	6440	6494	6030	740	70	134	1213	84	6	0.5

<sup>a</sup> Relative luciferase units.<sup>b</sup> Samples normalized for RNA content.<sup>c</sup> RNA concentrations of 1 µg per construct were added to HFF and BHK in vitro translation extracts.<sup>d</sup> RNA concentrations of 0.1 µg per construct were added to HFF and BHK in vitro translation extracts.

to associate with the 3'UTRs of certain flaviviruses (Blackwell and Brinton, 1997; De Nova-Ocampo et al., 2002) (Paranjape, S., Harris, E., unpublished results), may also play a role. It has been shown that the expression of certain translation factors, including eIF4E, eIF4G, and poly(A) binding protein, is upregulated in transformed cells (Gingras et al., 1999). Since the Nicaraguan DEN2 strains can be translated in transformed cell lines, i.e., HepG2 hepatoma cells and BHK (Diamond et al., 2000a), but not in HFF, critical translation factors may be limited in a primary cell such as HFF and less so in the transformed cell lines.

Previous sequence comparisons between DEN genotypes have shown that particular amino acid and nucleotide differences are associated with viral pathogenesis. Specifically, amino acid charge differences in the prM, E, NS3, NS4B, and NS5 genes have been associated with reduced pathogenicity in vivo (Ishak et al., 2001; Leitmeyer et al., 1999). In our study, consistent differences were also observed in prM, E, NS4B, and NS5. Our results demonstrated a small difference in binding ability between the Thai and Nicaraguan strains. These findings are consistent with recent studies that indicate that mutations in the DEN2 E protein and the UTRs together confer a specific replication phenotype (Cologna and Rico-Hesse, 2003). While the effects of a mutation in the prM or E protein may only be evident further upstream of viral translation (e.g., receptor affinity or neutralization in vivo), mutations in NS4B and NS5 may directly influence translation of the viral genome. For example, the NS5A protein of HCV was shown to play a role in the regulation of translation through the inhibition of interferon-stimulated protein kinase R, leading selectively to cap-independent translation (He et al., 2001). However, our assays detect a difference in efficiency at the step of translation of input strand viral RNA, even prior to the synthesis of the nonstructural viral proteins. In addition, we detect differences in the ability of RNA chimeric reporter constructs containing the DEN2 UTRs to be translated in the absence of viral proteins, indicating that the 3'UTR

alone can mediate certain aspects of translation efficiency. It is also possible that later steps in the viral life cycle contribute to the decreased infection efficiency of the low-passage Nicaraguan strains; for instance, these strains may be less efficient at RNA synthesis as well. Investigation of the effects of the specific differences in the DEN coding regions and the 3'UTR defined in this study is currently under way.

In conclusion, our results indicate that translation of genomic DEN2 RNA is a critical control point of viral replication in cells. Sequence differences in the 3'UTR that modulate translation efficiency correlate with replication phenotype of low-passage DEN2 strains. We show here that small variations in sequence can lead to significant differences in the ability of DEN viruses to productively infect cells and that these differences can be exploited to focus investigation on critical steps in the flavivirus life cycle, such as the mechanism of translation.

## Materials and methods

### *Cell culture, antibodies, and viral stocks*

Human foreskin fibroblasts (HFF), BHK-21 clone 15 (BHK), and C6/36 cells were cultured as previously described (Diamond et al., 2000a). The murine hybridomas used in this study produce antibodies directed against DEN envelope (E) protein of DEN2 (3H5-1) or DEN3 (5D4-11) (ATCC, Rockville, MD). Hybridoma culture, antibody purifications, immunoprecipitations, and immunostaining techniques were performed as previously described (Diamond et al., 2000a). DEN2 strains used in this study include a prototype DHF strain from Thailand (16681; provided by the Centers for Disease Control and Prevention, Fort Collins, CO), two recent DHF isolates from Thailand (C0477 and K0049; a gift from R. Rico-Hesse, San Antonio, TX), and DF isolates from the 1999 epidemic in Nicaragua

(N9622, N1042, N1043, N1047, and N1064; a gift from A. Balmaseda, Centro Nacional de Diagnóstico y Referencia, Managua, Nicaragua). All viruses belong to the Asian/American-Asian genotype of DEN2 (Balmaseda et al., 1999; Leitmeyer et al., 1999; Rico-Hesse et al., 1998). All experiments used viral stocks from tissue culture passage two, except those involving strain 16681, which is highly passaged (passage number unknown). Viral stocks were obtained as previously described (Diamond et al., 2000a).

#### *Plaque assay and cell infection*

Virus titers were determined by plaque assay using BHK cells as described previously (Diamond et al., 2000b). Virus concentrations were determined as plaque-forming units (PFU) per milliliter and used to calculate the multiplicity of infection (MOI) in experiments. Cells were infected according to previously described protocols (Diamond et al., 2000a). After infection, the cells were washed six times to remove residual virus and then incubated at 37°C for 48 to 72 hours (h) prior to harvest. At that time, viral supernatants were collected for plaque assay and cells were harvested for RNA quantitation.

#### *RNA extraction and quantitation*

Total RNA was extracted from equal numbers of infected cells using the RNeasy Minikit (Qiagen, Valencia, CA) and was eluted in 50  $\mu$ l of RNase-free water. Positive-strand DEN RNA was quantitated using a real-time RT-PCR assay with primers directed to the DEN2 3'UTR (Houng et al., 2000), Taqman reagents (One-Step RT-PCR Kit; Applied Biosystems, Foster City, CA), and an ABI PRISM 7700 sequence detection system (Applied Biosystems). A standard containing a known concentration of RNA transcribed from a plasmid containing the DEN2 3'UTR downstream of the T7 promoter was serially diluted to generate a five-point standard curve against which samples were measured.

In some experiments, the number of copies of viral RNA was measured using a previously described semiquantitative asymmetric competitive RT-PCR assay (Diamond et al., 2000a). Briefly, viral RNA was asymmetrically reverse transcribed using a primer directed against either the positive or the negative strand. The cDNA was then amplified by PCR, and the number of copies of viral RNA was measured relative to a 10-fold serially diluted standard of known concentration. The dilution at which the band of amplified DEN RNA appeared equivalent to the band of amplified standard was used as the concentration of DEN RNA initially added to the RT-PCR. RNA per cell was calculated as follows: RNA per cell = [(competitor concentration in copies per microliter)  $\times$  (total volume of RNA/volume of RNA in RT-PCR)]/(total number of cells).

#### *Binding assay*

Virus binding and entry in HFF cells were measured using a real-time RT-PCR assay (see above) (Diamond and Harris, 2001). Briefly, to measure binding, cells were incubated with DEN (MOI = 1) at 4°C for 2 h. After extensive washing to remove unbound virus, the amount of positive-strand RNA on the surface was quantitated by real-time RT-PCR. To measure virus entry, cells were incubated at 37°C for 2 h and then cooled to 4°C. Cells were then incubated with a 1 M NaCl solution at pH 9.5, which removes the surface bound without affecting internalized virus (Diamond and Harris, 2001), and the amount of positive-strand RNA within the cell was quantitated by real-time RT-PCR.

#### *Transfection of DEN RNA*

Infectious viral RNA was transfected into cells using DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol; Invitrogen, Carlsbad, CA). HFF or BHK cells were seeded at  $2 \times 10^5$  cells per well (70% confluence) in a 12-well plate and incubated at 37°C in 5% CO<sub>2</sub> overnight. Immediately prior to transfection, cell monolayers were washed with 2 ml of OptiMEM transfection medium (Invitrogen). In a polystyrene tube, 1 ml of OptiMEM was mixed with 16  $\mu$ l (for BHK) or 10  $\mu$ l (for HFF) of DMRIE-C reagent per well. In the same tube,  $1 \times 10^8$  copies of viral RNA per well, quantitated by competitive RT-PCR (Diamond et al., 2000a), were added and mixed thoroughly. The solution was added to each well, and cells were incubated at 37°C for 4 h. Cells were then washed twice with RPMI supplemented with 10% FBS to remove excess viral RNA and further incubated at 37°C for 48 h. Viral titer was determined via plaque assay of the supernatant. To determine transfection efficiency, cells were collected at 4 h post transfection, viral RNA was extracted using the RNeasy Minikit (Qiagen), and the amount of positive-strand RNA within the cell was quantitated by competitive RT-PCR (Diamond et al., 2000a).

#### *Translation assay and immunoprecipitation*

Translation of DEN RNA was visualized by labeling proteins with <sup>35</sup>S-cysteine-methionine (Amersham Biosciences, Piscataway, NJ) according to previously published methods (Baxt and Bablanian, 1976; Diamond et al., 2002). To immunoprecipitate viral proteins, HFF cells were seeded at  $1 \times 10^5$  cells/well in a 24-well plate, incubated overnight, and infected at an MOI of 100 with the DEN2 strains for 2 h at 37°C. The cells were washed three times and incubated in RPMI supplemented with 5% FBS until the specified time points. At 14 or 24 h post infection, the cells were incubated for 30 minutes (min) in RPMI cys<sup>-</sup>met<sup>-</sup> medium plus 5% dialyzed FBS. The cells were then incubated in 1.75-ml

RPMI cys<sup>-</sup>met<sup>-</sup> medium plus 0.5 mCi of <sup>35</sup>S-cysteine-methionine for 30 min. The cells were detached with HBSS plus 5 mM EDTA, washed once in cold PBS, and resuspended in 1 ml of lysis buffer (1% Triton X-100, 140 mM NaCl, 25 mM Tris-HCl, pH 7.8, 0.025% NaN<sub>3</sub>, 0.2 TIU/ml aprotinin, 2 mg/ml iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 0.5% bovine hemoglobin). Cells were lysed for 1 h at 4°C on a rotary shaker. Nuclear debris was removed by centrifugation at 3,000 × g for 5 min, and the membrane fraction was removed by microcentrifugation at 10,000 × g for 10 min. The lysate was precleared with bovine IgG Sepharose (200 μl, 10 mg/ml) for 4 h to overnight on a rotary shaker. Immunoprecipitation was performed overnight with anti-DEN2- or anti-DEN3-Sepharose (25 μl of a 2 mg/ml stock) in a volume of 500 μl [200 μl labeled lysate and 300 μl of 0.1% Triton X-100 supplemented with 140 mM NaCl, 25 mM Tris-HCl, pH 7.8, and 0.025% NaN<sub>3</sub> (TSA)] at 4°C. The beads were then washed three times with TSA plus 0.1% Triton X-100, once with TSA, and once with Tris-HCl, pH 6.8, at 4°C, and then resuspended in 20 μl of 5× SDS sample buffer. The immunoprecipitate was resolved on a 10% SDS gel and exposed to autoradiographic film.

To detect translation of input strand viral RNA, HFF cells were seeded and infected in the manner described above. The cells were washed three times and incubated in RPMI supplemented with 5% FBS until 4 or 24 h post infection. At these time points, the cells were incubated for 1 h in 500 μl of RPMI cys<sup>-</sup>met<sup>-</sup> medium with 5% dialyzed FBS. Thirty minutes prior to labeling, 175 mM NaCl was added to inhibit cellular but not viral translation (Erdei, 1981; Schrader and Westaway, 1990). <sup>35</sup>S-cysteine-methionine (100 μCi) was then added for 30 min to label newly synthesized proteins. The cells were detached with HBSS plus 5 mM EDTA, washed twice in cold PBS, and resuspended in 25 μl of SDS sample buffer containing 5% β-mercaptoethanol and 2 M urea. The labeled proteins were boiled and resolved on an 8% SDS-polyacrylamide gel, which was then exposed to a Molecular Dynamics PhosphorImager detection system (Amersham Biosciences). ImageQuANT Image Analysis software (Amersham Biosciences) was used to quantitate viral protein by measuring the area under the peak generated from a density profile of each protein band.

#### *Viral RNA synthesis assay*

DEN viral RNA synthesis was measured by metabolically labeling viral RNA in HFF cells with <sup>32</sup>P<sub>i</sub> according to a published method (Myers et al., 2001). HFF cells were seeded at 1 × 10<sup>6</sup> cells per flask 24 h prior to infection and incubated overnight to allow attachment. The cells were infected with DEN2 strains at an MOI of 5 and then starved in 5% PO<sub>4</sub><sup>-</sup>-deficient media [PO<sub>4</sub><sup>-</sup>-free Dulbecco's minimal essential media (DMEM), 5% DMEM, 5% dialyzed fetal bovine serum, 1% sodium pyruvate, 1 μg/ml actino-

mycin D] for 60 min prior to labeling. At specified time points post infection, 300 μCi/ml inorganic phosphoric acid [<sup>32</sup>P<sub>i</sub>] (ICN, Costa Mesa, CA) was added, and cells were incubated for 8 h. Cell monolayers were harvested using HBSS plus 0.025% trypsin. Viral RNA was extracted using the RNeasy Minikit (Qiagen) and resuspended in 30 μl of water. Viral RNA was separated by electrophoresis on a 0.7% formaldehyde gel and visualized by autoradiography.

#### *Sequence analysis and alignment*

DEN2 genomes were sequenced according to previously published methods (dos Santos et al., 2002). Total RNA was extracted from 500 μl of C6/36 cell culture supernatant by using the RNeasy Minikit (Qiagen). RNA was eluted in 50 μl of water, and 5 μl was used as a template for RT-PCR as described previously (Harris et al., 1998) with 30 cycles of amplification. Synthetic oligonucleotide primer pairs that generated 21 overlapping double-stranded DNA products spanning the DEN2 genome were used (dos Santos et al., 2002). For automated sequencing, spin column-purified (Qiagen) DNA fragments were prepared and analyzed according to Applied Biosystems 3100 Genetic Analyzer Systems specifications (Applied Biosystems, Foster City, CA).

Overlapping nucleic acid sequences were combined for analysis and edited with the aid of the GeneJockey II program (Biosoft, Cambridge, UK). DEN2 virus nucleic acid sequences were aligned with other DEN2 genomes using the DNAsis multiple-sequence alignment program (Hitachi Software Engineering Company, Ltd., Tokyo, Japan). The N1042 and C0477 DEN2 genomes sequenced in this study were compared with DEN2 genome sequences Thai 16681, Thai K0008, Thai K0010, and Brazil B64022, which were obtained from GenBank (accession numbers: U87411, AF100459, AF100460, and AF489932, respectively). Conserved amino acid or nucleotide differences were considered to be those that were common among all isolates within a strain but differed between the Thai and Nicaraguan/Brazilian strains. Amino acid changes displayed in Table 1 show only those residues presenting a charge change between strains. Targeted sequencing of specific regions to confirm conserved differences was performed by amplifying the indicated regions with the appropriate primers (dos Santos et al., 2002) using Nicaraguan isolates N1047 and N1043 as template.

#### *Reporter constructs*

DEN2 chimeric reporter constructs used in this study were derived from the pGL3-based 5DENLuc3DEN-16681 plasmid, which includes the 16681 5'UTR sequence, the first 72 nucleotides of the capsid region of 16681 fused to the firefly luciferase (Luc) gene, and the 16681 3'UTR sequence (Holden, K., Harris, E., unpublished data). To construct the 5DENLuc3DEN-Thai and 5DENLuc3DEN-Nica chimeras, the fragment between the *SgrAI* and the

*Xba*I restriction sites of 5DENLuc3DEN-16681 (encompassing the entire 3'UTR) was replaced with the corresponding fragment derived through splicing by overlap extension-PCR (SOE-PCR) fusion (Horton et al., 1989) of the 3' end of the luciferase gene with the 3'UTR of either the Thai (C0477) or the Nicaraguan (N1042) low-passage strains. RNA was transcribed from the T7 promoter using the RiboMAX Large Scale RNA Production System (Promega, Madison, WI).

#### *In vitro* translation assay

Translation of reporter constructs was evaluated in *in vitro* translation extracts generated from BHK or HFF cells using a combination of published methods (Bergamini et al., 2000; Favre and Trepo, 2001). BHK and HFF cells ( $2 \times 10^8$ ) were harvested, washed  $3 \times$  with PBS, and resuspended in 1 packed cell volume of translation buffer (20 mM HEPES, pH 7.4, 4 mM Mg-acetate, 5 mM DTT, 120 mM K-acetate, 100 mM sucrose, 1  $\mu$ M PMSF, 15 U aprotinin, 10 U leupeptin). Each sample was then lysed using a syringe with a 21-gauge needle and clarified by centrifugation ( $13,000 \times g$  for 5 min). Lysis and clarification were repeated once to ensure maximum cell disruption. Prior to translation, the cell extracts were supplemented with 5 mM spermidine (Sigma Chemical Co., St. Louis, MO), 20 mM creatine phosphate (Sigma), 40 U creatine kinase (Sigma), 40  $\mu$ M each amino acid (Promega), 2.5 mM ATP, 0.5 mM GTP, and 20 U Superasin (Ambion, Austin, TX). *In vitro* transcripts (1  $\mu$ g) of each construct described above were added to create a total volume of 50  $\mu$ l of the supplemented lysate. The lysates were incubated at 30°C for 60 min, and luciferase activity was measured using Luciferase Assay reagent (Promega) and a TD20/20 luminometer (Turner Designs, Sunnyvale, CA).

#### Northern blot

To test the stability of reporter constructs in *in vitro* translation assays, after a 60-min incubation in *in vitro* translation extracts, total RNA was extracted from 50  $\mu$ l of lysate by using the RNeasy Minikit (Qiagen). RNA was eluted in 30  $\mu$ l RNase-free water, and half of each sample was used per assay. Samples were placed in formaldehyde loading buffer, incubated at 68°C for 15 min, immediately cooled on ice, loaded onto a 1% agarose gel containing 2% formaldehyde, and electrophoresed. After transfer to a nylon membrane (Roche) overnight, samples were crosslinked by UV light. Subsequently, the membrane was preincubated in hybridization buffer (50% formamide,  $5 \times$  SSC, salmon sperm DNA, and  $5 \times$  Denhardt's blocking reagent) for 2 h at 60°C. The RNA probe containing  $\alpha$ - $^{32}$ P-labeled CTP, which was transcribed from a plasmid containing in the entire 3'UTR of the 16681 strain of DEN2, was added at a concentration of 50 ng of probe per milliliter of hybridization buffer directly to preincubated membrane solution and

hybridized overnight at 60°C. After hybridization, the membrane was washed twice with  $5 \times$  SSC plus 0.1% SDS at RT for 15 min and twice with  $0.5 \times$  SSC plus 0.1% SDS for 15 min at 60°C. RNA was visualized by exposure of membrane to radiographic film (BioMax MR, Kodak, Rochester, NY). Northern blot analysis was also performed in parallel with the biosynthetic labeling of DEN2 RNA to confirm the identity of the observed 11-kb band of RNA. HFF cells were seeded and infected in the manner of the viral RNA synthesis assay. Cells were harvested 24 h post infection and total RNA was extracted, electrophoretically separated, transferred to nylon membrane (Roche), and crosslinked as described above. To prepare membrane for hybridization of RNA probe, the membrane was preincubated in hybridization buffer (50% formamide, 0.02% SDS, 0.1% *N*-laurosarcosine, and  $5 \times$  SSC) plus 1% blocking reagent (Roche) for 2 h at 68°C. An RNA probe targeting the 453-nt DEN2 3'UTR was transcribed from the plasmid described above using digoxigenin-labeled nucleic acids (DIG) (Roche). The probe was added to preincubated membrane solution at a concentration of 50 ng of probe per milliliter of hybridization buffer and hybridized overnight at 68°C. DIG-labeled samples were visualized using DIG detection antibody (Roche) and the chemiluminescent substrate CDP-Star (disodium 2-chloro-5-(4-methoxy Spiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1<sup>3,7</sup>] decan}-4-yl)-1-phenyl phosphate) (Roche) according to manufacturer's instructions. RNA was visualized by exposure of membrane to radiographic film (Kodak).

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