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Enhancement of dengue virus translation: role of the 3′ untranslated region and the terminal 3′ stem-loop domain

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Abstract

An essential step for a productive infection by the dengue flavivirus (DEN) is translation of the m^7G-capped, nonpolyadenylated positive-sense RNA genome. We have recently identified sequences within the DEN 3′ untranslated region (UTR) that modulate viral translation. Here, we show that the DEN type 2 (DEN2) 3′UTR stimulated translation of m^7G-capped DEN2 5′UTR-containing reporter mRNAs in baby hamster kidney (BHK) cells compared to a 3′ vector sequence. Analogous to the 3′ poly(A) tail, the DEN2 3′UTR also enhanced translation of reporter mRNAs containing (i) a nonfunctional A cap, (ii) the 5′UTR of human β-globin, or (iii) a viral internal ribosome entry site (IRES). In all cases, approximately half of the translation efficiency was due to the terminal 3′ stem-loop (3′SL) domain. In addition, the 3′SL domain increased the association of mRNAs with polysomes. Together, these results indicate that the DEN2 3′UTR, mediated in part by the 3′SL domain, enhances translation initiation, possibly after recognition of the 5′ cap structure.

Keywords: Translational regulation; 3′ untranslated region; Cap-dependent translation initiation; Dengue virus; Flavivirus

Introduction

Dengue virus (DEN) causes significant morbidity and mortality in humans worldwide, ranging from the self-limiting dengue fever to the potentially life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). DEN is a member of the *Flavivirus* genus within the *Flaviviridae* family and is closely related to West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV). DEN exists as four serotypes (DEN1–4) and has an m^7G-capped, nonpolyadenylated positive-sense RNA genome of approximately 11 kilobases (Cleaves and Dubin, 1979; Wengler and Gross, 1978).

After entry into cells, the DEN genome is translated as a single polyprotein, which is cleaved into three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Hahn et al., 1988). Translation of the nonstructural proteins, a subset of which assembles into replication complexes, is essential for viral RNA synthesis (Khromykh et al., 2000). Unlike many other positive-strand animal viruses, flaviviruses do not shut off cellular protein synthesis (Westaway, 1973). Therefore, flaviviral RNA must compete for cellular translation machinery despite ongoing host mRNA translation. The RNA sequences of DEN that regulate this process are poorly defined, and the mechanism of viral translational regulation has not been established.

Eukaryotic and viral translation is often regulated at the rate-limiting step of initiation (Mathews et al., 2000). Cap-dependent initiation involves the recruitment of the 43S ribosomal subunit by the eukaryotic initiation factor 4F (eIF4F) complex, which binds to the m^7G cap (Gingras et al., 1999). The eIF4F complex is composed of a cap-binding protein (eIF4E), an RNA helicase

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(eIF4A), and a scaffold protein (eIF4G). After cap recognition, the small ribosomal subunit scans along the mRNA until it reaches an AUG start codon in the proper context; then the 60S subunit joins and peptide bond formation ensues (Kozak, 1989). An alternative mechanism of translation initiation involves internal ribosome entry sites (IRES), which directly recruit the 43S ribosome near the start codon, thus skipping any upstream AUGs (Jang et al., 1988; Pelletier and Sonenberg, 1988). Different classes of IRES have been shown to have different requirements for translation initiation factors, such as eIF4G, eIF4A, and eIF3 (Buratti et al., 1998; Pestova et al., 2001).

The 3′ ends of both viral and cellular mRNAs regulate translation initiation that proceeds by either cap-dependent or cap-independent mechanisms (Mazumder et al., 2003). The classic example is the 3′ poly(A) tail that follows the 3′ untranslated region (3′UTR) in most eukaryotic and viral mRNAs (Jackson and Standart, 1990). The 3′ poly(A) tail facilitates an interaction between the poly(A) binding protein (PABP) and the eIF4G component of eIF4F (Gallie and Tanguay, 1994; Tarun and Sachs, 1996) on either cap-dependent mRNAs (Tarun and Sachs, 1995) or IRES-containing mRNAs (Bergamini et al., 2000; Michel et al., 2001). The 3′ poly(A) tail has been proposed to (i) interact with eIF4F to recruit the 43S ribosome to the appropriate place at the 5′ end of the mRNA, (ii) increase the affinity of eIF4F for mRNA, (iii) stabilize the eIF4F complex, (iv) induce bound factors to allosterically stimulate other initiation factors, or (v) promote recycling of ribosomes back onto the mRNA (Sachs, 2000).

Similar to eukaryotes, viruses have evolved mechanisms to enhance viral translation via their 3′ ends. In the case of rotavirus mRNAs, which are capped and nonpolyadenylated, a viral protein (NSP3) interacts with both the viral 3′ terminal sequences and eIF4G, resulting in an enhancement of translation (Chizhikov and Patton, 2000; Piron et al., 1998; Vende et al., 2000). Barley yellow dwarf virus (BYDV) is another example of viral translation regulated by the 3′UTR. BYDV lacks both a 5′ cap and a 3′ poly(A) tail, and stimulates translation through an RNA–RNA interaction between the 5′ and 3′UTRs (Guo et al., 2001). We hypothesized that the DEN 3′UTR affects viral protein synthesis in an analogous manner to these and other viral 3′UTRs, helping the DEN RNA to compete with cellular mRNAs for the host translation machinery. Consistent with this, we identified sequences within the DEN2 3′UTR that modulate viral translation and infectivity (Edgil et al., 2003), thus implicating 3′UTR in the regulation of viral translation.

Here, we initiated an investigation into the mechanism by which the DEN2 3′UTR modulates translation, focusing on the role of the 5′ cap structure and the 5′UTR sequence. Addition of the DEN2 3′UTR downstream of firefly luciferase (LUC) stimulated translation of reporter mRNAs containing different 5′UTRs and 5′ cap structures as compared to addition of a 3′ vector sequence. Translational stimulation by the DEN2 3′UTR was comparable to that imparted by the 3′ poly(A) tail. The role of the highly conserved, terminal 3′ stem-loop (3′SL) (Hahn et al., 1987; Mohan and Padmanabhan, 1991) was also assessed. The 3′SL is essential for replication of the viral genome and for virion production (You and Padmanabhan, 1999; Zeng et al., 1998), and has been implicated in regulation of WNV translation (Li and Brinton, 2001). Approximately half of the translation efficiency of the DEN2 3′UTR was attributable to the terminal 3′SL domain. In summary, we show that the DEN2 3′UTR is a translational enhancer acting independently of the 5′ cap and the 5′UTR sequence, and that the 3′SL accounts for half of the translational activity.

Results

The DEN2 3′UTR stimulates translation

The DEN UTRs regulate both viral translation and RNA synthesis; therefore, reporter mRNAs that do not encode the DEN replicase were utilized to measure the effect of the DEN2 UTRs on translation alone. A DEN2 reporter mRNA was generated consisting of the DEN2 5′UTR and 72 nt of the viral capsid coding sequence upstream of luciferase (LUC) and the 451 nt DEN2 3′UTR downstream of LUC (Fig. 1A, "5′DEN-LUC-3′DEN"). The 5′ end of capsid was included in the DEN2 reporter mRNA because it contains the conserved 5′ cyclization sequence (5′CD) (Hahn et al., 1987). The 5′CD is complementary to the 3′ cyclization domain (3′CD) in the 3′UTR, and the interaction of these two elements is predicted to circularize the RNA genome (Hahn et al., 1987) and is critical for viral replication (You and Padmanabhan, 1999). A control reporter mRNA in which the DEN2 3′UTR was replaced with 268 nt of vector sequence (Fig. 1A, "5′DEN-LUC-3′V268") was constructed. The 5′ ends of the mRNAs were capped with m^7GpppA cap analog during in vitro transcription. These DEN2 reporter mRNAs were transfected separately into baby hamster kidney (BHK) cells for 1 h, and luciferase activity was monitored at several time points up to 24 h after the initiation of transfection.

The 5′DEN-LUC-3′DEN mRNA consistently generated higher luciferase activity than the 5′DEN-LUC-3′V268 mRNA starting at 2 h post-transfection. Little luciferase activity was observed at 1 h, while the peak of luciferase activity occurred between 4 and 8 h post-transfection (Fig. 2A; data in figures are representative of several experiments). Four hours post-transfection was chosen for analysis of translation efficiency because this was before significant mRNA degradation occurred (see LUC mRNA $t_{1/2}$ in Table 1; data in tables show averages of at least four

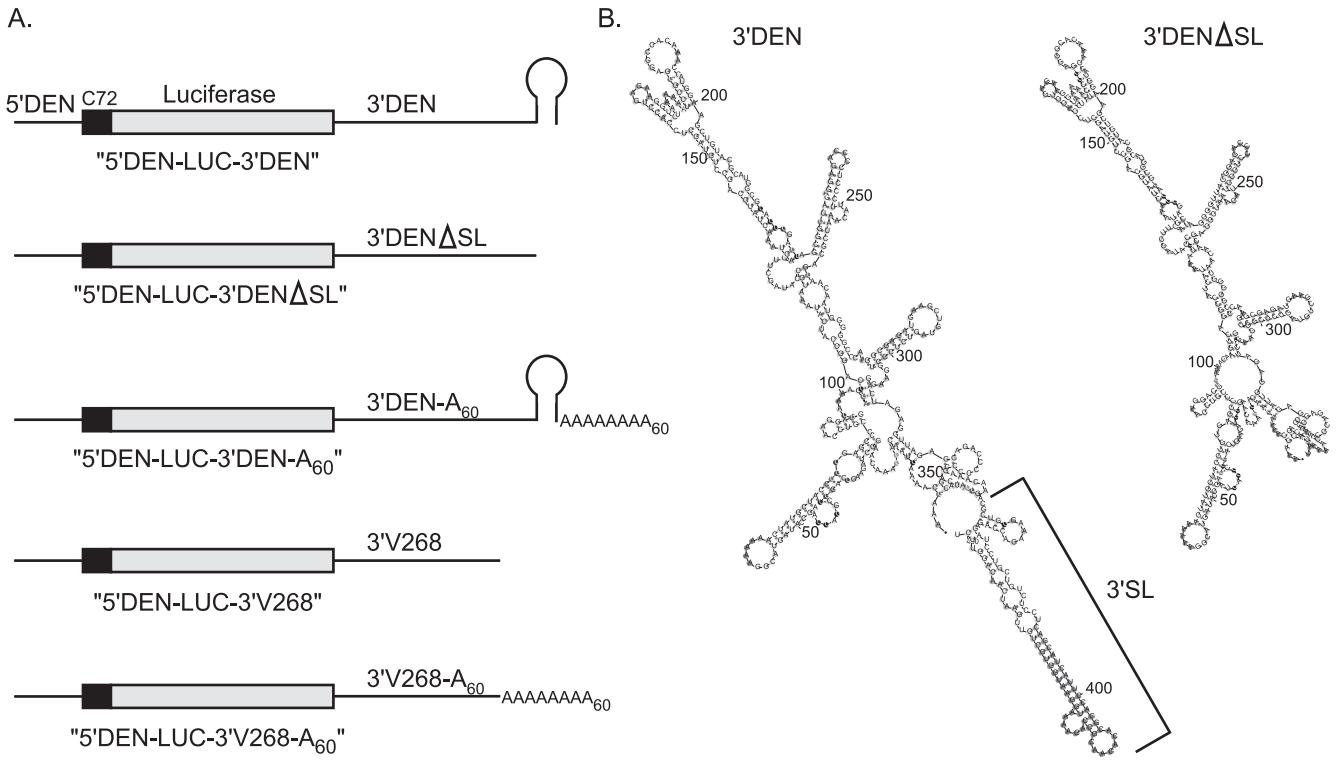
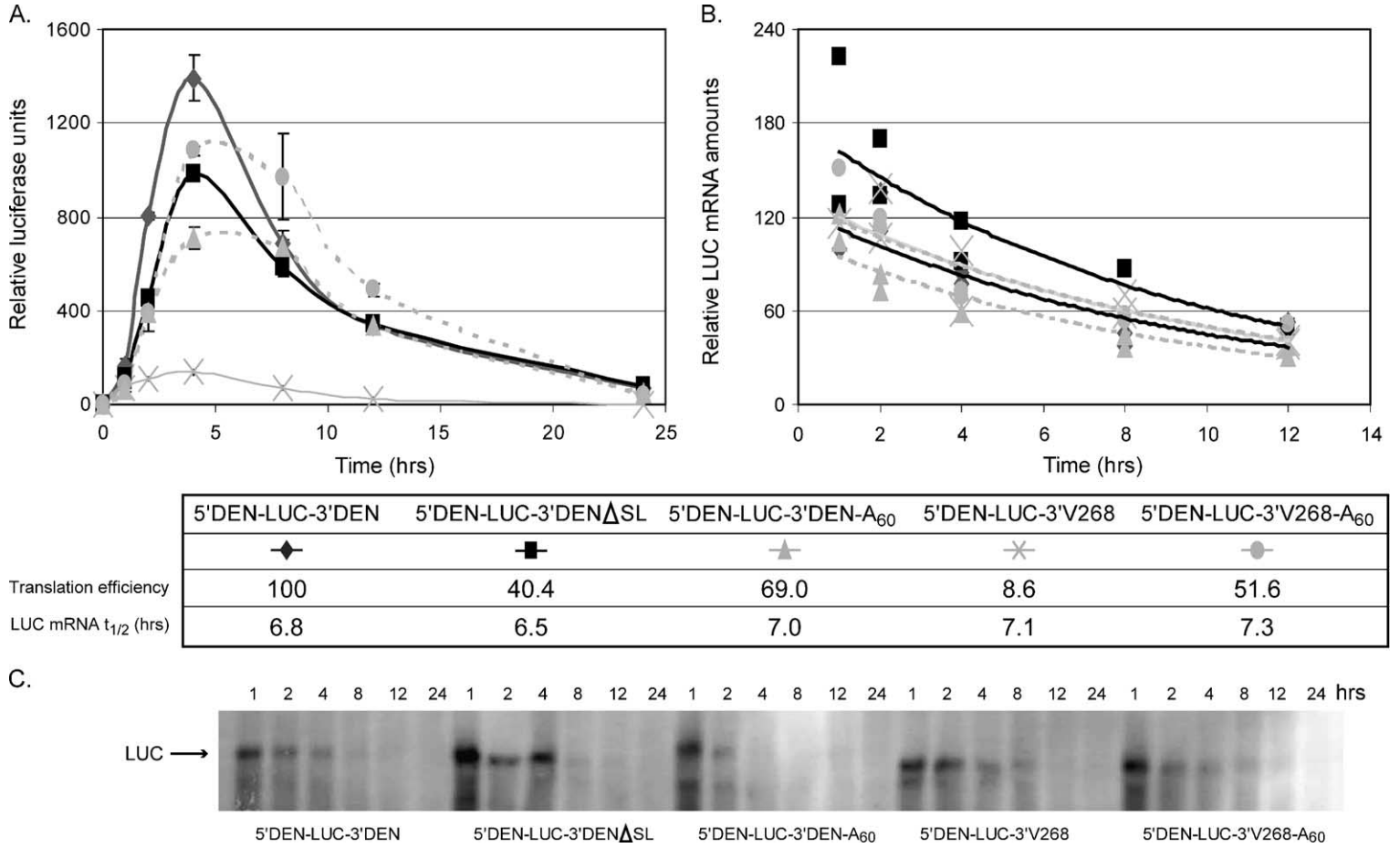


Fig. 1. (A) Schematic diagram of the DEN2 luciferase reporter mRNAs. Shown are mRNAs with the 5′UTR of DEN2 ("5′DEN") and the first 72 nt of capsid coding sequence ("C72", black box) followed by the LUC gene (gray box). Downstream of LUC is either the DEN2 3′UTR ("5′DEN-LUC-3′DEN"), the DEN2 3′UTR without the terminal 3′SL domain ("5′DEN-LUC-3′DENΔSL"), the DEN2 3′UTR followed by a 3′ poly(A) tail ("5′DEN-LUC-3′DEN-A_{60}"), a vector sequence ("5′DEN-LUC-3′V268"), or the vector sequence followed by a 3′ poly(A) tail ("5′DEN-LUC-3′V268-A_{60}"). (B) Predicted secondary structure of the DEN2 3′UTR with and without the 3′SL domain as determined by the *mfold* web server under standard conditions (Zuker, 2003). The 3′SL is indicated in the structure of the full-length DEN2 3′UTR, as is the nucleotide position from the start of the DEN2 3′UTR in both structures.

experiments) and while luciferase activity was increasing linearly (Fig. 2A). Relative luciferase activity was normalized to the amount of reporter mRNA present when transfection reagent was removed 1 h after its addition to the cells to account for any differences in transfection efficiency within each experiment. Transfection efficiency varied randomly between different reporter mRNAs in separate experiments, indicating that mRNA degradation did not account for these differences. In addition, LUC mRNA was normalized to the amount of β-actin mRNA as an internal control. After normalizing for mRNA transfection efficiency, the translation efficiency of mRNAs with the full-length DEN2 3′UTR was used as a baseline to which to compare the other reporter mRNAs. For example, in the experiment shown in Fig. 2, the 5′DEN-LUC-3′V268 reporter mRNAs were translated at approximately 9% of the level of the 5′DEN-LUC-3′DEN reporter mRNAs.

The relative translation efficiency of the DEN2 3′UTR compared to the 3′ vector sequence was consistent across experiments (Table 1), although variability in relative luciferase units between independent experiments was observed, likely due to differences in cellular conditions upon transfection. Overall, the translation efficiency of the 5′DEN-LUC-3′V268 mRNA was approximately 10% of that of the 5′DEN-LUC-3′DEN mRNA (Table 1), after normalizing for transfection efficiency. Moreover, similar results were obtained in human (Hep3B) and mosquito (C6/36) cell lines (data not shown), indicating that the effect of the DEN2 3′UTR on translation can occur in various cell types.

To determine whether the stimulatory effect of the DEN2 3′UTR on translation was due to differences in mRNA stability, the mRNA half-life and integrity of each reporter mRNA were determined using RNA harvested over a 12-h period. The average stability ($t_{1/2}$ = 5.5 h for 5′DEN-LUC-3′DEN and 6.6 h for 5′DEN-LUC-3′V268) (Table 1) and quality of full-length LUC mRNA (Fig. 2C) were similar for the two reporter mRNAs as determined by real-time RT-PCR and Northern blot hybridization, respectively. Complete degradation of the full-length transcript was apparent 24 h post-transfection by Northern blot analysis (Fig. 2C). The stability and amount of each reporter mRNA as determined by real-time RT-PCR were similar to the results obtained by semiquantitative Northern analysis (data not shown), suggesting that real-time RT-PCR analysis was sufficient for determining mRNA stability. In short, these results demonstrate that mRNA stability does not account for the effect of the DEN2 3′UTR on luciferase expression.



	5'DEN-LUC-3'DEN	5'DEN-LUC-3'DENΔSL	5'DEN-LUC-3'DEN-A_{60}	5'DEN-LUC-3'V268	5'DEN-LUC-3'V268-A_{60}
	◆	■	▲	✳	●
Translation efficiency	100	40.4	69.0	8.6	51.6
LUC mRNA $t_{1/2}$ (hrs)	6.8	6.5	7.0	7.1	7.3

Fig. 2. Relative translation efficiency of m^7G-capped DEN2 reporter mRNAs in BHK cells. (A) Relative luciferase units were determined at 1, 2, 4, 8, 12, and 24 h after RNA transfection into BHK cells of m^7G-capped 5′DEN-LUC-3′DEN (dark gray diamonds), 5′DEN-LUC-3′DENΔSL (black squares), 5′DEN-LUC-3′DEN-A_{60} (gray triangles), 5′DEN-LUC-3′V268 (gray X's), or 5′DEN-LUC-3′V268-A_{60} (gray circles). Error bars indicate the standard deviation of the mean for duplicate samples. (B) Relative LUC mRNA amounts of duplicate samples were determined by real-time RT-PCR analysis of LUC-containing mRNAs in transfected cells at the indicated time points. Exponential trend lines have an $R^2 \geq 0.70$. The table displays the translation efficiency at 4 h post-transfection and the mRNA half-life of each reporter mRNA for this experiment. The relative translation efficiency was calculated by dividing RLU at 4 h by the amount of transfected mRNA at 1 h (at the completion of transfection). The value for 5′DEN-LUC-3′DEN was set to 100 as the baseline. The half-life of each reporter mRNA was calculated as described in Materials and methods. (C) Northern blot of the m^7G-capped mRNAs in BHK was probed using a DIG-labeled LUC probe. Representative data from at least seven independent experiments are shown, except that the translation efficiency for "5′DEN-LUC-3′V268-A_{60}" was abnormally low in this experiment as compared to the average (see Table 1).

Table 1
Relative translation efficiency and mRNA half-life of reporter mRNAs containing an m^{7}G-capped DEN2 5′UTR

Reporter mRNA	Translation efficiency[a]	LUC mRNA $t_{1/2}$ (h)
5′DEN-LUC-3′DEN	100	5.5 ± 0.9
5′DEN-LUC-3′DENΔSL	54 ± 8**	5.5 ± 0.7[b]
5′DEN-LUC-3′DEN-A_{60}	176 ± 43[b]	6.6 ± 0.8[b]
5′DEN-LUC-3′V268	13 ± 5**	6.6 ± 0.8[b]
5′DEN-LUC-3′V268-A_{60}	222 ± 40**	5.8 ± 0.8[b]

** $P < 0.01$ when compared to mRNAs containing the full-length DEN2 3′UTR.
[a] For each reporter mRNA, RLU at 4 h was adjusted to the amount of transfected LUC mRNA. Translation efficiency was determined relative to mRNAs containing the full-length DEN2 3′UTR. The average translation efficiency and LUC mRNA half-life and their associated standard deviation of the mean were calculated from at least seven experiments.
[b] Not significantly different from mRNAs containing the full-length DEN2 3′UTR.

The DEN2 3′UTR and a 3′ poly(A) tail each stimulate translation of DEN2 5′UTR reporter mRNAs

The DEN genomic RNA lacks a 3′ poly(A) tail (Cleaves and Dubin, 1979), which acts as a translational enhancer for other mRNAs (Jackson and Standart, 1990). To test if a 3′ poly(A) tail could stimulate translation of reporter mRNAs that contain the DEN2 5′UTR, a synthetic poly(A) tail of 60 nt, which is sufficient to stimulate translation (Bergamini et al., 2000; Preiss et al., 1998), was incorporated into the DEN2 mRNA reporter construct downstream of the 3′ vector control sequence (Fig. 1A). On average (n = 14), the "5′DEN-LUC-3′V268-A_{60}" mRNA demonstrated approximately 20-fold greater translation relative to the 5′DEN-LUC-3′V268 mRNA after normalizing for transfection efficiency (Table 1), indicating that the 3′ poly(A) tail can function with the DEN2 5′UTR. Real-time RT-PCR and Northern blot analysis confirmed equivalent mRNA stability between the reporter mRNAs (Table 1; Fig. 2C). Therefore, either the DEN2 3′UTR or a synthetic 3′ poly(A) tail can stimulate translation of DEN2 5′UTR-containing mRNAs, although a 3′ poly(A) tail may be more efficient.

In general, translation of mRNAs with a 3′ poly(A) tail is significantly greater than mRNAs that lack a 3′ poly(A) tail (Gallie and Tanguay, 1994; Tarun and Sachs, 1996). We sought to determine if the DEN2 3′UTR and a 3′ poly(A) tail could enhance translation in an additive manner, as this would provide information as to whether the two 3′ sequences act by a similar or distinct mechanism. To address the question, a "5′DEN-LUC-3′DEN-A_{60}" reporter mRNA was generated (Fig. 1A). The relative amount of this reporter mRNA as well as its half-life was found to be similar to other constructs following transfection (Fig. 2B; Table 1). On average, the addition of the 3′ poly(A) tail to the end of the DEN2 3′UTR appeared to increase translation only 2-fold as compared to 5′DEN-LUC-3′DEN, although there were some inconsistencies between experiments (Table 1; Fig. 2). This is significantly less than the increase in translation when the 3′ poly(A) tail is added after the 3′ vector control sequence (approximately 20-fold) (Table 1), but is similar to the average translation efficiency obtained for 5′DEN-LUC-3′V268-A_{60} (Table 1). Thus, the DEN2 3′UTR and a 3′ poly(A) tail can replace each other to enhance translation of DEN2 reporter mRNAs, but they do not appear to act in a strongly additive or cooperative manner.

The conserved terminal 3′ stem-loop domain contributes to DEN2 3′UTR translational enhancement

The terminal sequence of the DEN2 3′UTR, as well as that of all other flaviviral 3′UTRs, forms a conserved, stable stem-loop (3′SL) structure (Hahn et al., 1987; Mohan and Padmanabhan, 1991), which is important for viral RNA synthesis and virion production (You and Padmanabhan, 1999; Zeng et al., 1998). To determine whether the region encompassing the DEN2 3′SL plays a role in translation, the entire 3′SL domain (the terminal 98 nt) was deleted to generate the "5′DEN-LUC-3′DENΔSL" reporter mRNA (Fig. 1A). This 3′SL deletion had little predicted effect on upstream secondary structures (Fig. 1B), as calculated using the *mfold* web server (Zuker, 2003). In the experiment shown in Fig. 2 and in replicate experiments (Table 1), the 5′DEN-LUC-3′DENΔSL mRNA displayed approximately half of the luciferase activity as 5′DEN-LUC-3′DEN mRNA after adjusting the relative luciferase activity to the amount of transfected mRNA. Reporter mRNA stability did not account for the differences in translation efficiency at 4 h (Fig. 2; Table 1). Thus, the DEN2 3′SL domain is necessary for maximal translation mediated by the DEN2 3′UTR, but not for mRNA stability.

The DEN2 3′UTR, partially through the 3′SL domain, enhances translation of mRNAs independently of the 5′ cap structure

We next sought to determine whether translational enhancement by DEN2 3′UTR depends on the m^{7}G(5′)ppp(5′)N cap (m^{7}G cap), because the DEN2 genomic RNA contains an m^{7}G cap (Cleaves and Dubin, 1979; Wengler and Gross, 1978). The m^{7}G cap, present on the 5′ end of most eukaryotic mRNAs, prevents mRNA degradation and recruits eIF4E, the cap-binding factor of eIF4F, to initiate translation. A nonfunctional A(5′)ppp(5′)N cap (A cap) prevents RNases from degrading the 5′ end of mRNAs, but cannot recruit eIF4E efficiently (Bergamini et al., 2000). Before examining the requirement of a functional m^{7}G cap structure for the DEN2 3′UTR to stimulate translation, it was necessary to first assess whether the 5′ cap on the reporter mRNAs both stabilizes the mRNA and functionally recruits eIF4E as expected. A set of DEN2 5′UTR-containing reporter mRNAs was transcribed in vitro without a cap, with an A cap, or with an m^{7}G cap. In BHK cells, maximal translational

activity was obtained with m^{7}G-capped reporter mRNAs, A-capped mRNAs displayed intermediate levels of translation, and uncapped mRNAs were translated the least efficiently (Fig. 3). The average half-lives of m^{7}G-capped, A-capped, and uncapped mRNAs were different, in that m^{7}G-capped mRNAs were the most stable and A-capped mRNAs were more stable than uncapped mRNAs (Table 2). Therefore, these data suggest that the relative translational activity of uncapped, A-capped, and m^{7}G-capped mRNAs is due to the differential ability of these mRNAs to recruit eIF4F via the cap-binding protein eIF4E and to differences in mRNA stability.

Next, the different 5′ cap structures were used to test whether the DEN2 3′UTR requires a functional 5′ cap structure to enhance translation (Fig. 3). The DEN2 3′UTR slightly stimulated translation of uncapped mRNAs (2-fold over 5′DEN-LUC-3′V268) (Table 2). In contrast, 5′DEN-LUC-3′DEN mRNAs containing the nonfunctional A cap or the functional m^{7}G cap were translated approximately 20- and 10-fold better than 3′ vector-containing mRNAs, respectively (Table 2). Once again, the DEN2 3′SL domain was necessary for maximal translation, as it accounted for approximately 50% of the translation of uncapped, A-capped, and m^{7}G-capped 5′DEN-LUC-3′DEN mRNAs (Table 2). As a control, it was determined that a 3′ poly(A) tail increased translation compared to mRNAs lacking a poly(A) tail by approximately 4-fold for uncapped mRNAs, 6-fold for A-capped mRNAs, and 20-fold for m^{7}G-capped mRNAs (Table 2). mRNA stability did not account for the differences in translation within sets of uncapped, A-capped, or m^{7}G-capped mRNAs (Table 2). Therefore, the DEN2 3′UTR, similar to a 3′ poly(A) tail, stimulates translation irrespective of the presence of a functional m^{7}G cap, and the 3′SL region contributes to this stimulatory activity.

The DEN2 3′UTR via the 3′SL domain augments translation of either cap-dependent or IRES-containing reporter mRNAs

The DEN2 3′UTR could require a direct interaction with the DEN2 5′UTR to enhance translation and therefore may not be able to stimulate translation of mRNAs that contain a non-DEN 5′UTR. To test whether the DEN2 3′UTR stimulates translation of mRNAs with other 5′UTRs, reporter mRNAs were constructed with the human β-globin 5′UTR upstream of LUC, and, downstream of LUC, either the DEN2 3′UTR (“5′βg-LUC-3′DEN”), the DEN2 3′UTR lacking the 3′SL domain (“5′βg-LUC-3′DENΔSL”), the vector control sequence (“5′βg-LUC-3′V268”), or the vector sequence with a 3′ poly(A) tail (“5′βg-LUC-3′V268-A$_{60}$”) (Fig. 4A). The 5′UTR of β-globin was chosen because translation initiation of m^{7}G-capped β-globin mRNA is cap-dependent, thus requiring the complete eIF4F complex to recruit the 43S ribosomal subunit (Lockard and Lane, 1978).

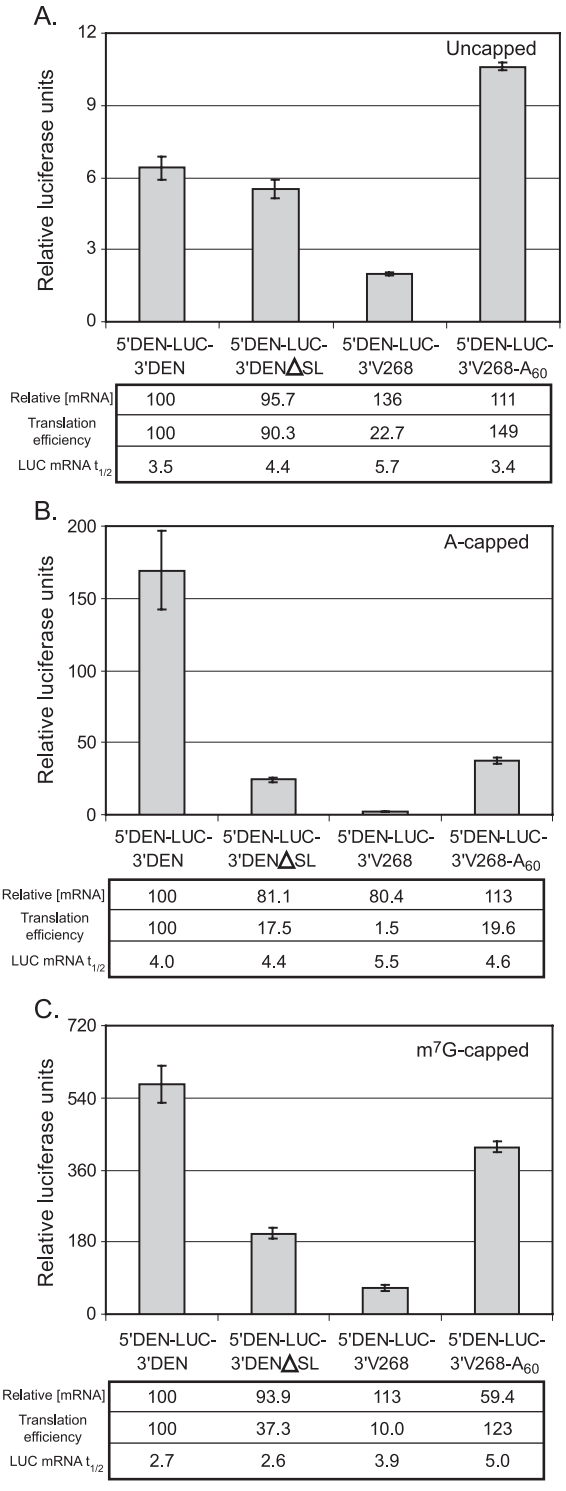


Fig. 3. Translation efficiency of DEN2 reporter mRNAs with different 5′ cap structures. BHK cells were transfected with 5′DEN-LUC-3′DEN, 5′DEN-LUC-3′DENΔSL, 5′DEN-LUC-3′V268, or 5′DEN-LUC-3′V268-A$_{60}$ mRNAs with either no cap (A), an A cap (B), or an m^{7}G cap (C) at the 5′ end, as indicated. Cells were harvested in duplicate to monitor luciferase activity at 4 h post-transfection. Error bars indicate the deviation of the mean for duplicate samples. Relative RNA transfection efficiency at 1 h, relative translation efficiency at 4 h, and the mRNA half-life of each reporter mRNA for this experiment are indicated below the graph (see Fig. 2). Results from a single experiment are shown as representative of at least four independent experiments.

Table 2
Relative translation efficiency of reporter mRNAs with different 5′ cap structures on the DEN2 5′UTR

Reporter mRNA	5′ cap structure	Translation efficiency[a]	LUC mRNA $t_{1/2}$ (h)[a]
5′DEN-LUC-3′DEN	no cap	100	3.7 ± 0.6
5′DEN-LUC-3′DENΔSL	no cap	68 ± 9*	4.4 ± 0.9[b]
5′DEN-LUC-3′V268	no cap	39 ± 12**	4.3 ± 0.8[b]
5′DEN-LUC-3′V268-A_{60}	no cap	170 ± 37[b]	4.2 ± 1.0[b]
5′DEN-LUC-3′DEN	A	100	4.0 ± 0.5
5′DEN-LUC-3′DENΔSL	A	33 ± 8**	4.2 ± 0.8[b]
5′DEN-LUC-3′V268	A	5.1 ± 1.2**	5.6 ± 1.4[b]
5′DEN-LUC-3′V268-A_{60}	A	30 ± 9*	4.6 ± 0.1[b]
5′DEN-LUC-3′DEN[c]	m^7G	100	5.5 ± 0.9
5′DEN-LUC-3′DENΔSL[c]	m^7G	54 ± 8**	5.5 ± 0.7[b]
5′DEN-LUC-3′V268[c]	m^7G	13 ± 5**	6.6 ± 0.8[b]
5′DEN-LUC-3′V268-A_{60}[c]	m^7G	222 ± 40**	5.8 ± 0.8[b]

[a] For each reporter mRNA, RLU at 4 h was adjusted to the amount of transfected LUC mRNA. Translation efficiency was determined relative to mRNAs containing the full-length DEN2 3′UTR within each set. The average translation efficiency and LUC mRNA half-life and their associated standard deviation of the mean were calculated from at least four experiments.
[b] Not significantly different from mRNAs containing the full-length DEN2 3′UTR.
[c] These data are also presented in Table 1.
* $P < 0.05$ when compared to mRNAs containing the full-length DEN2 3′UTR.
** $P < 0.01$ when compared to mRNAs containing the full-length DEN2 3′UTR.

As a control for translational enhancement using β-globin reporter mRNAs, a 3′ poly(A) tail was found to increase translation of reporter mRNAs on average 9-fold compared to the vector sequence alone (Table 3). Similarly, the DEN2 3′UTR increased translation of reporter mRNAs 6-fold compared to the 3′ vector sequence (Table 3), indicating that the DEN2 3′UTR can enhance translation of mRNAs that contain cap-dependent 5′UTRs other than that of DEN2. Translation of 5′βg-LUC-3′DENΔSL reporter mRNAs was reduced nearly 60% compared to translation of full-length DEN2 3′UTR-containing mRNAs (Table 3). Transfection efficiency and average mRNA stability did not account for the differences in translation (Fig. 4A; Table 3). Therefore, the 3′SL domain is necessary for maximal translation efficiency of mRNAs containing a distinct cap-dependent 5′UTR, while direct interaction between the DEN2 5′ and 3′UTRs does not appear to be essential for the DEN2 3′UTR to enhance translation.

Thus far, our results indicated that the DEN2 3′UTR can stimulate translation of reporter mRNAs independently of both a functional m^7G cap and the DEN2 5′UTR. The next question was whether enhancement of translation by the DEN2 3′UTR could be mediated by a cap-independent mechanism (e.g., through an IRES), as this may provide further insights into the requirements of the DEN2 3′UTR to enhance translation. Reporter mRNAs containing the

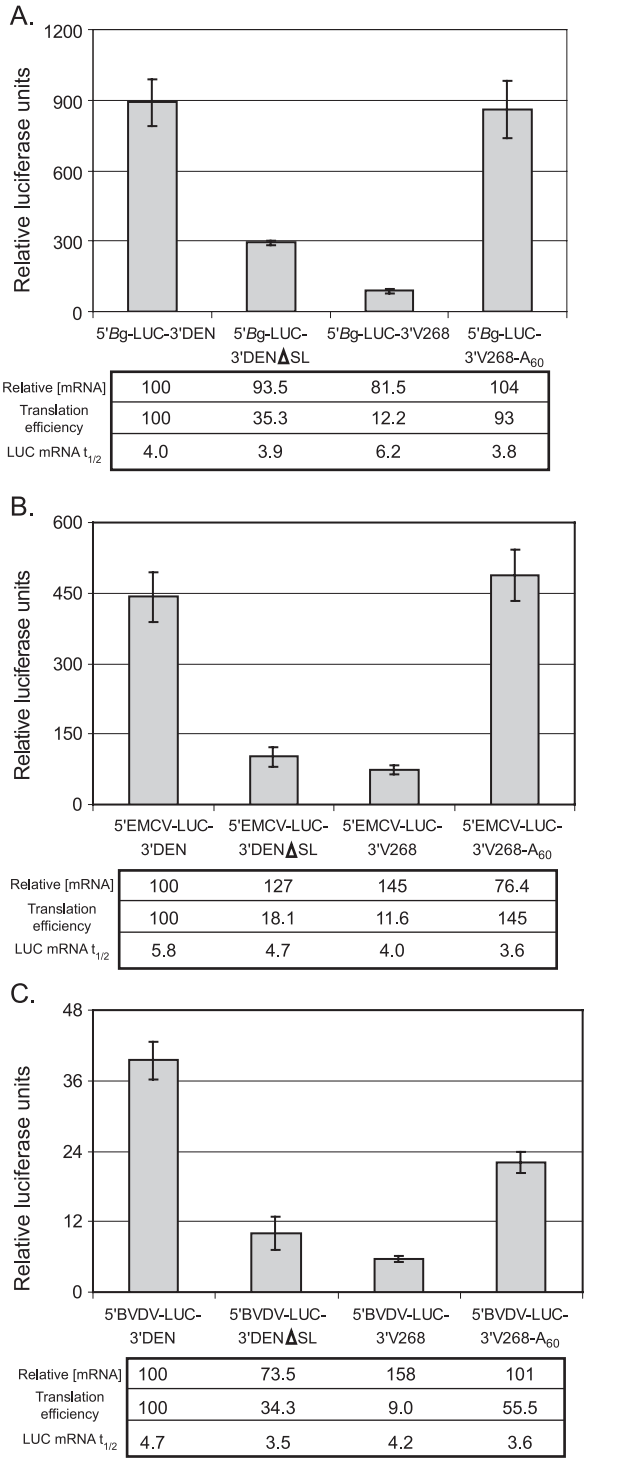


Fig. 4. Translation efficiency of reporter mRNAs with nonflaviviral 5′UTRs. BHK cells were transfected with reporter mRNAs containing the following 5′UTRs: 5′β-globin (A), 5′EMCV (B), or 5′BVDV (C). Reporter mRNAs contained either the DEN2 3′UTR, the 3′DENΔSL, a 3′ vector sequence of 268nt, or the vector sequence with a 3′ poly(A) tail, as indicated. Cells were harvested in duplicate to monitor luciferase activity 4 h after the start of transfection. Error bars indicate the standard deviation of the mean for duplicate samples. LUC mRNA transfection efficiency, relative translation efficiency at 4 h, and the half-life of each reporter mRNA for this experiment are shown below the graph (see Fig. 2). Data shown are from a single experiment as representative of at least five independent experiments.

Table 3
Relative translation efficiency of reporter mRNAs containing different 5′UTRs

Reporter mRNA	5′ cap structure	Translation efficiency[a]	LUC mRNA $t_{1/2}$ (h)[a]
5′βg-LUC-3′DEN	m^7G	100	5.8 ± 1.7
5′βg-LUC-3′DENΔSL	m^7G	43 ± 12**	4.1 ± 1.5[b]
5′βg-LUC-3′V268	m^7G	16 ± 3**	5.4 ± 0.8[b]
5′βg-LUC-3′V268-A_{60}	m^7G	143 ± 14*	7.1 ± 2.0[b]
5′EMCV-LUC-3′DEN	A	100	5.9 ± 0.9
5′EMCV-LUC-3′DENΔSL	A	29 ± 9**	3.8 ± 0.6[b]
5′EMCV-LUC-3′V268	A	23 ± 4**	3.7 ± 0.2[b]
5′EMCV-LUC-3′V268-A_{60}	A	180 ± 36[b]	7.2 ± 1.6[b]
5′BVDV-LUC-3′DEN	A	100	4.9 ± 0.5
5′BVDV-LUC-3′DENΔSL	A	36 ± 6**	4.6 ± 1.0[b]
5′BVDV-LUC-3′V268	A	12 ± 3**	5.0 ± 1.5[b]
5′BVDV-LUC-3′V268-A_{60}	A	177 ± 67[b]	3.2 ± 0.4[b]

[a] For each reporter mRNA, RLU at 4 h was adjusted to the amount of transfected LUC mRNA. Translation efficiency was determined relative to mRNAs containing the full-length DEN2 3′UTR within each set. The average translation efficiency and LUC mRNA half-life and their associated standard deviation of the mean were calculated from at least five experiments.
[b] Not significantly different from mRNAs containing the full-length DEN2 3′UTR.
* $P < 0.05$ when compared to mRNAs containing the full-length DEN2 3′UTR.
** $P < 0.01$ when compared to mRNAs containing the full-length DEN2 3′UTR.

5′UTRs of EMCV and BVDV were constructed to test this hypothesis (Figs. 4B and C). These viruses each contain an IRES that directly recruits the 43S ribosome for translation in a cap-independent manner. The IRES of EMCV requires eIF4G, eIF4A, and eIF3 to initiate translation (Kolupaeva et al., 1998; Lomakin et al., 2000), whereas the BVDV IRES functions independently of these factors, requiring only the 43S ribosomal complex and eIF3 (Pestova and Hellen, 1999). The mRNAs for the EMCV and BVDV constructs were synthesized with a nonfunctional A cap to increase the stability of these mRNAs and thus the relative luciferase activity. Nonetheless, similar results were obtained with uncapped IRES-containing reporter mRNAs (data not shown).

In BHK cells, A-capped 5′EMCV-LUC-3′V268-A_{60} and 5′BVDV-LUC-3′V268-A_{60} mRNAs displayed on average 8- and 15-fold higher levels, respectively, of translation than mRNAs that lacked the 3′ poly(A) tail (Table 3), indicating that the translation of either IRES could be stimulated by a 3′ poly(A) tail as expected. Likewise, the DEN2 3′UTR stimulated translation of reporter mRNAs containing the EMCV or BVDV IRES approximately 4- and 8-fold, respectively, compared to the 3′ vector control sequence (Table 3). Translation of both 5′EMCV-LUC-3′DENΔSL and 5′BVDV-LUC-3′DENΔSL mRNAs was reduced more than 60% compared to that of 5′EMCV-LUC-3′DEN and 5′BVDV-LUC-3′DEN (Table 3), respectively. Thus, analogous to the results with the β-globin 5′UTR, the 3′SL domain was necessary for optimal translation of IRES-containing LUC mRNAs. The different 3′ ends did not affect mRNA stability of the reporter mRNAs, as determined by real-time RT-PCR (Table 3). These data demonstrate that the DEN2 3′UTR, partly through its 3′SL domain, can stimulate translation of mRNAs that contain either a cap-dependent 5′UTR or cap-independent IRES.

The DEN2 3′SL domain stimulates translation at the step of initiation

That the DEN2 3′UTR, in part through the 3′SL domain, can enhance both cap-dependent and cap-independent translation suggests that the DEN2 3′UTR may function late in initiation (at a stage common to the different 5′UTRs), during elongation, or at termination. To determine the step of translation facilitated by the DEN2 3′UTR through the 3′SL region, the association of reporter mRNAs with polysomes was analyzed by sucrose gradient fractionation (Fig. 5A). As an internal control for each transfected reporter mRNA, the association of endogenous GAPDH mRNA with polysomes was also assessed. No significant difference in polysome association of GAPDH mRNAs was found (Fig. 5C). Compared to m^7G-capped 5′DEN-LUC-3′DENΔSL mRNAs, the full-length DEN2 3′UTR-containing reporter mRNAs were translated more efficiently and were associated with polysomal fractions to a greater degree (Figs. 5B and C). Comparison of the ratio of the 5′DEN-LUC-3′DEN to 5′DEN-LUC-3′DENΔSL reporter mRNAs in each polysome fraction demonstrated that 5′DEN-LUC-3′DEN mRNAs were associated with heavier polysomes than mRNAs lacking the 3′SL domain (Fig. 5D). These results suggest that the region that includes the 3′SL enhances translation by both increasing the amount of mRNAs recruited into polysomes and the number of ribosomes associated with the mRNA, and, therefore, may be increasing the rate of translation initiation.

It is possible, however, that the effect of the 3′SL domain on polysome association is not due to increased initiation, but is due to either an increase in the recruitment of mRNAs out of a nontranslatable pool or an increase in mRNP association, which would increase the rate of sedimentation independently of translation. Cycloheximide (CHX), which inhibits peptide bond formation, does not affect the association of mRNAs with mRNPs, nor does it release mRNAs from a nontranslatable region of the cell. Thus, in the presence of CHX, initiating mRNAs finish the initiation step of translation and are stalled in elongation, which results in a build-up of these mRNAs in polysomal fractions (Fig. 6A) (Lodish, 1971). In the following experiments, CHX was used to determine how the 3′SL region affects the sedimentation profiles of reporter mRNAs.

CHX was added when transfection of either 5′DEN-LUC-3′DEN or 5′DEN-LUC-3′DENΔSL reporter mRNAs was complete. Luciferase activity was greatly reduced in the presence of CHX, indicating that translation was inhibited

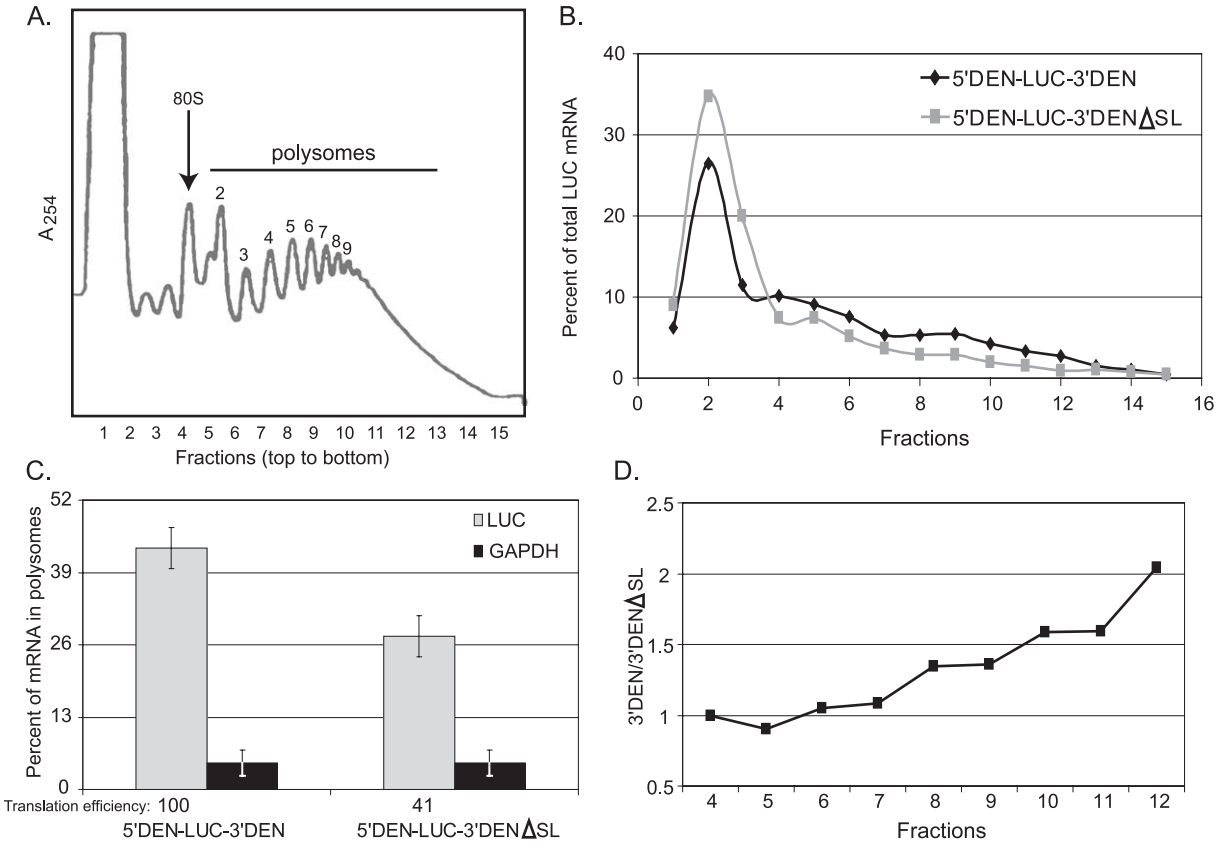


Fig. 5. Association of DEN2 reporter mRNAs with polysomes. BHK cells were transfected with different reporter mRNAs and subjected to sucrose gradient centrifugation and fractionation while A_{254} was monitored. Total RNA was extracted from each fraction and subjected to real-time RT-PCR to detect LUC mRNAs. (A) A representative A_{254} profile from BHK cells is shown, with the location of the 80S ribosome and the polysomes indicated. (B) Percentage of m^7G-capped 5′DEN-LUC-3′DEN or 5′DEN-LUC-3′DENΔSL reporter mRNA per fraction was plotted. (C) Shown is the percentage of LUC and GAPDH mRNA associated with polysome fractions (#5–12) from different mRNA transfections. The difference in association with polysomes between 5′DEN-LUC-3′DEN and 5′DEN-LUC-3′DENΔSL is significantly different ($P < 0.01$). An average from at least three independent experiments is shown with the error bars indicating the standard deviation of the mean. Average translation efficiency of the different reporter mRNAs from this series of experiments is indicated below the graph. (D) The ratio of the amount of 5′DEN-LUC-3′DEN versus 5′DEN-LUC-3′DENΔSL within each polysome fraction was calculated. In A, B, and D, representative data from at least three independent experiments are shown.

effectively (Fig. 6B). The effect of CHX on 5′DEN-LUC-3′DEN reporter mRNAs associating with polysomes was minor (Fig. 6B), possibly because a high percentage of these mRNAs was already in the elongation phase at the time of CHX addition. In contrast, the 5′DEN-LUC-3′DENΔSL reporter mRNAs increased their association with polysomes in the presence of CHX (Fig. 6B). Many of these mRNAs were not in the elongation phase when CHX was added, so these mRNAs were available to continue through initiation and to recruit ribosomes in the presence of CHX. Similar results were obtained when CHX was added to cells before RNA transfection (data not shown). A cellular mRNA was used as a control for differences in mRNA transfection and in CHX addition, and it was determined that for each transfection, β-actin mRNA, which is predominantly associated with polysomes in the absence of CHX, was unaffected by CHX treatment (Fig. 6C). The CHX results indicate that differences in polysome association between mRNAs containing or lacking the 3′SL region may be due to differences in translation initiation and not due to either mRNP association or recruitment from a nontranslatable pool. Therefore, the presence of the 3′SL domain seems to increase mRNA association with polysomes by increasing initiation efficiency.

Discussion

The DEN2 3′UTR stimulates translation of mRNAs that contain the DEN2 5′UTR as well as other cellular and viral 5′UTRs that initiate translation via either a cap-dependent or cap-independent mechanism. The DEN2 3′UTR also stimulates translation independently of a functional 5′ cap structure, consistent with its ability to enhance translation of mRNAs containing an IRES. Together, these data suggest that the DEN2 3′UTR stimulates translation at a stage common to cap-dependent and IRES-mediated initiation, and may act after recognition of the 5′ cap structure by

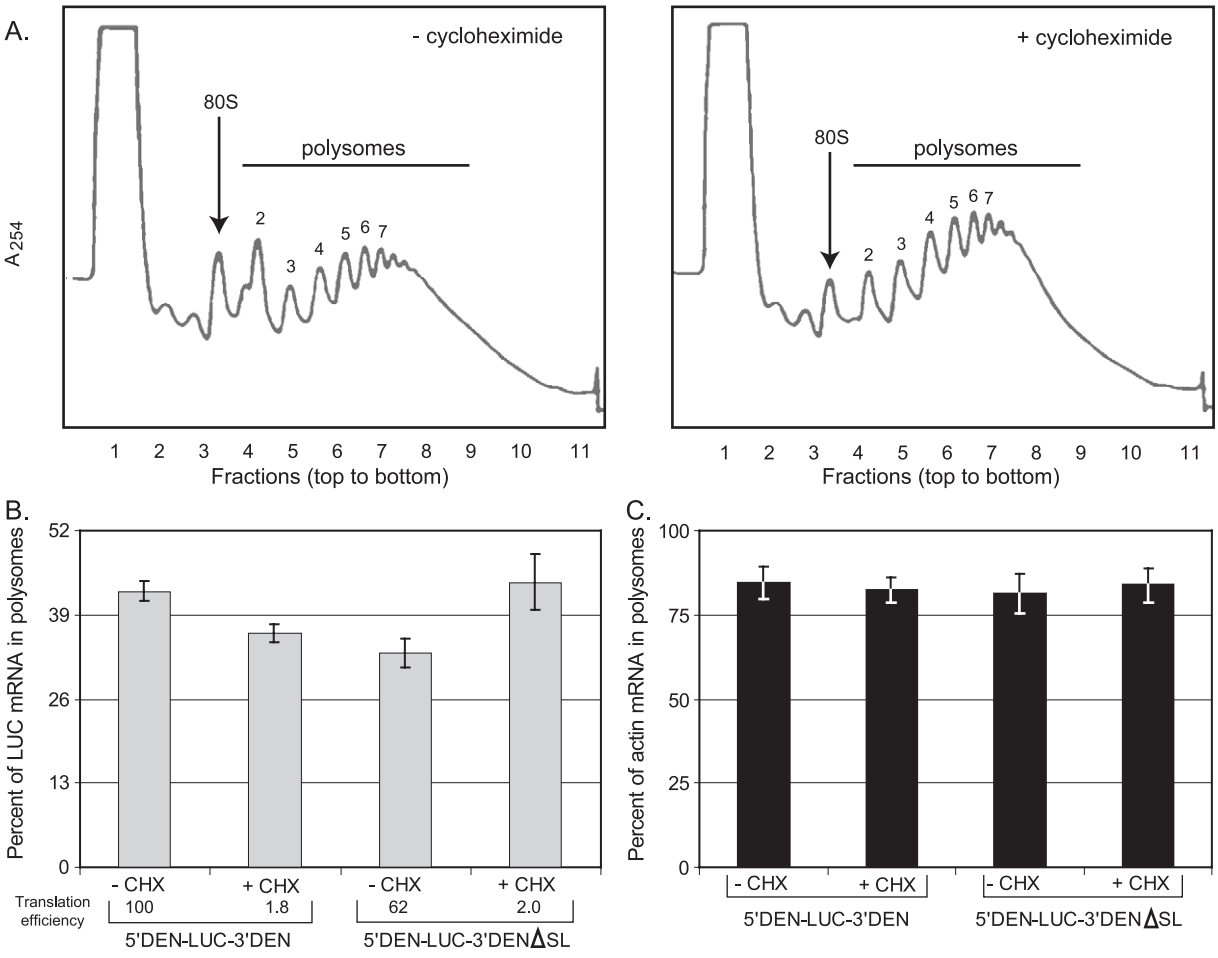


Fig. 6. Association of DEN2 reporter mRNAs with polysomes in the presence of cycloheximide. Transfected BHK cells were treated with CHX 1 h before sucrose gradient analysis. (A) A representative profile of polysomes from BHK cells with or without CHX treatment is shown. The location of the 80S ribosome and the polysomes is indicated. (B) The percentage of 5′DEN-LUC-3′DEN and 5′DEN-LUC-3′DENΔSL mRNAs associated with polysomes (#4–11) in the presence and absence of CHX was averaged over several experiments. Indicated below the graph is the average translation efficiency for these mRNAs. (C) Percentage of β-actin mRNA, as an internal control, associated with polysomes with and without CHX treatment for the different transfections was determined. In B and C, error bars indicate the standard deviation of the mean of several experiments.

eIF4F. The region that includes the terminal 3′SL contributes significantly to DEN2 3′UTR-mediated translation and appears to facilitate initiation, as the 3′SL domain increases polysome formation. Therefore, the DEN2 3′UTR likely enhances translation after recognition of the 5′ cap structure, in part due to an increase in translation initiation mediated by the 3′SL domain.

Our data indicate that the DEN2 3′UTR, through the 3′SL domain, is functionally analogous to a eukaryotic 3′ poly(A) tail, in that they both appear to facilitate translation during initiation and are independent of the 5′ cap structure for their activity (Bergamini et al., 2000; Munroe and Jacobson, 1990). Thus, it is probable that, like the 3′ poly(A) tail, the DEN2 3′UTR enhances translation by facilitating recruitment of factors involved in translation initiation. Because the DEN2 3′UTR and the synthetic 3′ poly(A) tail after the DEN2 3′UTR did not appear to act in a strongly additive or cooperative manner in our reporter mRNA experiments, it is possible that these elements bind proteins that act at the same step of translation initiation or that sterically interfere with each other, thereby preventing additional enhancement of translation.

In most of the experiments, mRNAs with a 3′ poly(A) tail translated up to twice as efficiently as mRNAs with the DEN2 3′UTR. The greater translation efficiency of the 3′ poly(A) tail may be attributable to the specialization of the 3′ poly(A) tail in upregulating translation of most cellular mRNAs by both stabilizing the mRNA and recruiting initiation factors. In contrast, the stimulation of translation efficiency imparted by the DEN2 3′UTR may be constrained because the DEN2 3′UTR must also participate in negative-strand viral RNA synthesis. Alternatively, the virus may have evolved to be translated less efficiently than polyadenylated mRNAs to balance viral translation and RNA synthesis or to avoid induction of an antiviral response. It is unknown why flaviviruses lack a 3′ poly(A) tail, but we have shown that the DEN2 3′UTR acts in an analogous manner to a 3′ poly(A) tail to increase translation. Therefore, a 3′ poly(A) tail may no

longer be essential for flaviviral RNAs to be competitive in a cellular environment with an excess of polyadenylated mRNAs.

Here we demonstrate that, in addition to its role in viral RNA replication and virus production (You and Padmanabhan, 1999; Zeng et al., 1998), the terminal 3′SL sequence or structure positively regulates DEN2 translation, as deletion of the 3′SL domain consistently decreased the translation efficiency of DEN2 3′UTR-containing mRNAs. This contrasts with an earlier report, in which the 3′SL of WNV was found to moderately inhibit translation of a reporter mRNA when placed downstream of a 3′ vector sequence in BHK cells (Li and Brinton, 2001). Significant differences in the experimental design between the WNV 3′SL paper and our study on the DEN2 3′SL may account for these differing results. Additionally, the WNV 3′SL was examined alone, downstream of a vector sequence, whereas sequences or structures in the rest of the flaviviral 3′UTR may be important for the 3′SL to regulate translation by providing a proper context.

The role of the DEN2 3′UTR in stimulating translation was not affected by removal of the 5′ cyclization domain (data not shown), a conserved sequence that is essential for replication (You and Padmanabhan, 1999) and that is involved in circularization of the genome via interaction with a complementary sequence in the 3′UTR (Hahn et al., 1987). In addition, the 3′SL domain still contributed half of the translational efficiency of DEN2 3′UTR-containing reporter mRNAs that lacked the 5′ cyclization domain (data not shown). Thus, circularization of the DEN genome via the cyclization domains does not appear to be necessary for translational enhancement mediated by the DEN2 3′UTR.

The DEN2 3′UTR appears to stimulate translation differently from other nonpolyadenylated viral 3′UTRs. Specifically, the 3′UTR of rotavirus mRNAs depends not only on viral NSP3 proteins to bind to both a viral 3′UTR and eIF4G (Piron et al., 1998; Vende et al., 2000), but also on a functional m^7G cap for translational enhancement (Chizhikov and Patton, 2000). In contrast, the DEN2 3′UTR can enhance translation of mRNAs with a nonfunctional 5′ cap structure and in the absence of viral proteins. However, the mechanisms of translational enhancement adopted by rotavirus and DEN are similar in that they both utilize the terminal 3′ end of an m^7G-capped viral mRNA and both can stimulate the translation of mRNAs independently of the sequence of the 5′UTR (Chizhikov and Patton, 2000). This mechanism differs from that of the nonpolyadenylated BYDV 3′UTR, which interacts directly with the BYDV 5′UTR to enhance translation via “kissing stem-loops” (Guo et al., 2001), thus requiring a specific sequence at the 5′ end of the mRNA. Therefore, the DEN2 3′UTR acts differently than other viral 3′UTRs in that it can stimulate translation independently of the 5′ cap structure and the 5′UTR sequence and in the absence of viral proteins.

The mechanism of translational stimulation by DEN2 3′UTR is also comparable to the translational enhancement mediated by the terminal 3′ stem-loop structure of histone mRNAs (Gallie et al., 1996). Similar to DEN2 RNAs, histone mRNAs have an m^7G cap and are nonpolyadenylated. The histone 3′ stem-loop structure recruits stem-loop binding protein (SLBP) (Dominski et al., 1995), which facilitates an interaction with translation initiation factors (eIF4E, eIF4G, and eIF3) and the m^7G cap (Ling et al., 2002). The DEN 3′SL domain may be acting in a similar manner to the histone 3′ stem-loop to mediate upregulation of translation, in that both may recruit translation initiation factors to stimulate translation at initiation.

Enhancement of translation by DEN2 3′UTR might be modulated by the presence of viral proteins, although this was not directly addressed using our translation reporter system. An on-going DEN2 infection had no effect on the translation of m^7G-capped 5′DEN-LUC-3′DEN mRNAs (data not shown), suggesting that viral proteins do not affect first-round translation. It is possible that viral proteins, or virally induced cellular proteins, further enhance translation after RNA replication. These proteins could also facilitate a switch between translation and RNA replication, because the DEN RNA is both an mRNA and a template for negative-strand RNA synthesis. These two processes occur in opposite directions on the same positive-sense genomic RNA (Barton et al., 1999; Gamarnik and Andino, 1998). Because there is evidence that the 3′SL functions at both translation and viral RNA replication (You and Padmanabhan, 1999; Zeng et al., 1998), it may be involved in the switch from one process to the other, depending on which proteins are bound to the 3′UTR.

Because neither viral proteins nor a direct RNA–RNA interaction between the 5′ and 3′UTRs appear to be required for translational enhancement by the DEN2 3′UTR, our results suggest that cellular proteins bound to the DEN2 3′UTR may modulate this effect. Several cellular proteins have been shown to bind to the 3′SL of WNV (Blackwell and Brinton, 1995, 1997), the 3′SL of JEV (Ta and Vrati, 2000), and the 3′UTR of three DEN serotypes (Blackwell and Brinton, 1995; De Nova-Ocampo et al., 2002; Garcia-Montalvo et al., 2004; S. Paranjape and E. Harris, unpublished results), yet whether these proteins regulate flavivirus translation is unknown. We are currently identifying the cellular proteins that regulate DEN2 translation through interaction with the DEN2 3′UTR and host translation machinery. Additional research is essential to elucidate the mechanism of translational enhancement by the DEN2 3′UTR and the switch between viral protein synthesis and viral RNA replication. In addition to uncovering unique mechanisms of eukaryotic translational regulation, this information may aid in the development of antiviral therapies that preferentially inhibit flaviviral translation within an infected cell.

Materials and methods

Cell lines

Baby hamster kidney (BHK) cells (ATCC, Manassas, VA) were cultured in αMEM supplemented with 2 mM L-glutamine (Gibco BRL, Carlsbad, CA) and 10% fetal bovine serum (Omega Scientific, Tarzana, CA) at 37 °C and 5% CO_2. Cells were passaged every 3–4 days using HBSS with 3 mM EDTA and 10 mM HEPES to suspend the monolayer.

Construction of luciferase reporter constructs

The infectious clone of DEN2 strain 16681, pD2/IC (gift of R. Kinney, CDC, Fort Collins, CO), was used as a template to construct the DEN2 mRNA reporter constructs. The T7 promoter and 5′UTR plus the first 72 nt of capsid coding sequence were amplified using primers PD2IC-F and 5′DEN*Nco*I-R (see supplementary data for a table of primer sequences). All primers were obtained from QIAGEN (Valencia, CA). PFU turbo polymerase (Stratagene, La Jolla, CA) and Taq polymerase (Roche, Mannheim, Germany) were used in the PCR reactions according to the manufacturer's instructions. PCR products were gel-extracted using QIAquick Gel Extraction Kit (Qiagen) and cloned using TA TOPO Cloning Kit (Invitrogen, Carlsbad, CA). The cloned PCR products were excised using the restriction enzymes *Mlu*I and *Nco*I, ligated into pGL3-Basic (Promega, Madison, WI) using T4 DNA ligase HC (Invitrogen), and transformed into STBL2 Ca^{2+} competent cells (Invitrogen). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). The resulting plasmid (pGL3–5′DEN-LUC) was isolated using the QIA-filter midi kit (Qiagen) and sequenced by the University of California at Berkeley DNA sequencing facility.

Splicing by overlap extension (SOE)-PCR (Warrens et al., 1997) was performed to fuse the 3′UTR of DEN2 to the 3′ end of LUC. First, the 3′LUC-F and LUC3′DEN-R primers were used to amplify the 3′ end of LUC from pGL3-Basic; the second PCR amplified the 3′UTR of DEN2 from pD2/IC using the 3′DEN-F and the 3′DEN*Xba*I-R primers. To perform SOE-PCR, 1 μl of each PCR product was used as template, along with 0.5 μM of each outside forward and reverse primer (3′LUC-F and 3′DEN*Xba*I-R). The resulting PCR product was cloned into pGL3–5′DEN-LUC using *Sgr*AI and *Xba*I to generate the pGL3–5′DEN-LUC-3′DEN plasmid. The pGL3–5′DEN-LUC-3′DENΔSL construct was generated by replacing the DEN2 3′UTR with a PCR product generated by using the 3′LUC-F and 3′DENΔSL*Xba*I-R primers.

The human β-globin 5′UTR was generated by SOE-PCR using PD2IC-F and 5′βg(A1G)-R primers with pD2/IC as template in the first PCR and primers 5′βgLUC-F and 5′LUC-R with pGL3-Basic as template in the second PCR. The resulting SOE-PCR product was inserted into pGL3-Basic using *Mlu*I and *Bst*BI to generate the pGL3–5′βg-LUC construct. The first nucleotide of the 5′UTR of β-globin was changed from an A to G to increase in vitro RNA synthesis by the T7 promoter (Imburgio et al., 2000). Again, the 3′UTR of DEN2 or the 3′DENΔSL was cloned into pGL3–5′βg-LUC, as described above, to generate pGL3–5′βg-LUC-3′DEN or pGL3–5′βg-LUC-3′DENΔSL, respectively.

The templates for amplifying the BVDV 5′UTR (pBVDV/IRES) and the EMCV 5′UTR (pR/dEMCV/F) were provided by A. Gamarnik (Fundacíon Campomar, Argentina) and R. Donis (University of Nebraska, Lincoln, NE), and by P. Sarnow (Stanford University, CA), respectively. The BVDV IRES was amplified using 5′BVDV-F and 5′BVDV*Nco*I-R primers, and the EMCV IRES was amplified using the primers 5′EMCV-F and 5′LUC-R. These products were cloned into pGL3-Basic using *Mlu*I and either *Nco*I or *Bst*BI to generate pGL3–5′BVDV-LUC and pGL3–5′EMCV-LUC, respectively. The full-length DEN2 3′UTR or the 3′DENΔSL was cloned into these plasmids, as above.

A synthetic poly(A) tail of 60 nt was added to the end of pGL3–5′DEN-LUC-3′DEN by digesting with *Xba*I, followed by treatment with T4 DNA polymerase (NEB) and subsequent digestion with *Sal*I. The pGL3 construct was ligated to the synthetic poly(A) tail excised from *Sma*I- and *Sal*I-digested pGLO18A (gift from A. Miller, Iowa State University, IW). To insert the synthetic poly(A) tail after 3′ vector sequence, the pGL3-based constructs were digested with *Bam*HI instead of *Xba*I, and then processed as described above.

In vitro transcription and RNA transfection

Luciferase reporter constructs were linearized with *Bam*HI (3′ vector control constructs), *Xba*I (DEN2 3′UTR or 3′DENΔSL constructs, leaving only 4 nt of vector sequence at the very 3′ end), or *Ase*I (3′ poly(A) constructs, leaving 5 nt of vector sequence after 60 As). After digestion, plasmid DNA was separated on an agarose gel and extracted using QIAquick Gel Extraction Kit (Qiagen). For in vitro transcription, 1 μg of linearized DNA template was used to program RiboMax Large Scale RNA Production System using T7 RNA polymerase (Promega). Cap analogs m^7GpppN (NEB), ApppG (NEB), or ApppA (Sigma, St. Louis, MO) were included in the reaction to incorporate different 5′ cap structures into the mRNA during in vitro transcription. m^7GpppA and ApppA were used with the constructs containing the DEN2 5′UTR, which begins with an A at the +1 position. All other constructs begin with a G at the +1 position for transcription, so m^7GpppG or ApppG cap analogs were used. After a 4-h incubation at 37 °C, samples were treated with DNase I (Promega) for 15 min at 37 °C, and unincorporated nucleotides were removed using NucAway Spin Columns (Ambion, Austin, TX). The RNA was quantified by a

spectrophotometer (Ultrospec 1000, Pharmacia Biotech, England).

For standard RNA transfection experiments, BHK cells were seeded (2×10^5 cells/well) in 12-well plates or, alternatively, in 24-well plates (1×10^5 cells/well), and incubated overnight at 37 °C with 5% CO_2. LipofectAMINE2000 (Invitrogen) was mixed with 3 or 6 pmol of RNA (for 24- and 12-well plates, respectively), and cells were transfected according to the manufacturer's instructions. Equimolar amounts of RNA were utilized, and no reproducible difference in transfection efficiency was found (Figs. 2–5). Transfection was stopped by washing the cells 1 h after addition of the RNA-LipofectAMINE2000 mixture. Cells were harvested at several time points starting at the end of the transfection period (e.g., 1 h after transfection commenced), as indicated in the figure legends, using HBSS/EDTA solution. Half of each sample was lysed for luminometry using Cell Culture Lysis Reagent (CCLR) (Promega), and the other half was treated with TRIZOL (Invitrogen) for extracting total RNA. All cell lysates and RNA samples were stored at −80 °C until processed. To perform luciferase assays, 10 μl of lysate was mixed with 50 μl Luciferase Assay Reagent (Promega). The samples, diluted 1:10 in CCRL if necessary, were analyzed using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA) with a 3-s delay and a 15-s read.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using TRIZOL-extracted total RNA samples and LUX Fluorogenic Primers (Invitrogen). The target sequence, near the 3′ end of LUC, was amplified using a FAM-labeled forward primer (5′-GAC CTT GTG GAC GAA GTA CCG AAA GG[FAM]C-3′) and an unlabeled reverse primer (5′-GCC CTT CTT GGC CTT TAT GAG-3′). As an internal control, the mouse/rat GAPDH or β-actin JOE-labeled LUX primer sets (Invitrogen) were used. SuperScript II Platinum One-Step Quantitative RT-PCR System (Invitrogen) and an ABI PRISM 7700 (Applied BioSystems, Foster City, CA) were used according to the manufacturer's protocols. A standard curve was generated by diluting a mixture of LUC mRNA and total RNA extracted from BHK cells.

Northern hybridization

Digoxigenin (DIG)-labeled RNA probes for detecting LUC mRNA were transcribed in vitro using *Bcl*I-linearized pSP-*luc*$^+$ plasmid (Promega) or pTRI-28S (Ambion) and DIG RNA Labeling Mix (Roche). Total RNA (0.5 μg) after TRIZOL extraction was separated by electrophoresis on a formaldehyde gel for 3 h at 70 V. The RNA was transferred to a positively charged nylon membrane (Roche) by capillary action. Hybridization with the DIG RNA probe was carried out according to the manufacturer's instructions. Anti-DIG alkaline phosphatase antibody and NBT/BCIP (Roche) were used to detect the hybridized probe by a colorimetric reaction.

Sucrose gradient centrifugation and fractionation

Confluent BHK cells in a T150 flask were split 1:6 and were grown overnight at 37 °C and 5% CO_2. Cells were washed with media and then transfected with 75 pmol of RNA using LipofectAMINE2000 (Invitrogen). Cells were returned to the incubator for two more hours and then removed from the flasks with HBSS/EDTA solution. Cells were washed twice, an aliquot was removed for luminometry, and the rest were lysed with a polysome lysis buffer (Hensold et al., 1996) on ice for 5 min with occasional vortexing. Cellular debris was pelleted by centrifugation at 6000 rpm for 5 min at 4 °C. The supernatants were loaded on 10–50% step sucrose gradients and were subjected to ultracentrifugation at 39 000 rpm in an SW41 rotor for 65 min at 4 °C. The gradients were fractionated using an ISCO gradient fractionator with an attached absorbance monitor reading at 254 nm. Fractions were frozen in liquid N_2 and stored at −80 °C. Total RNA was extracted using TRIZOL (Invitrogen). For inhibition of translation at elongation, 10 μg/ml of cycloheximide (CHX) (Sigma) was added to cells 30 min post-transfection, after removal of the transfection reagent. Cells were harvested for polysome analysis 1 h after CHX addition.

Statistical analysis and mRNA half-life calculations

Luciferase activity was normalized for each set of reporter constructs by dividing the value at each time point by the amount of transfected mRNA present at 1 h (when transfection reagent was removed), as detected by real-time RT-PCR. The amount of transfected reporter mRNA was normalized to the amount of β-actin mRNA as an internal control. To calculate relative translation efficiency, the activity of the reporter mRNA with the full-length DEN2 3′UTR was used as a baseline set at 100, because there was variability in relative luciferase units between experiments. The half-life of each mRNA was determined by plotting the relative amount of each mRNA, determined by real-time RT-PCR, versus time. An exponential curve was determined using Excel (Microsoft Corporation), and only the equations ($y = me^{-bx}$) with an R^2 value greater than 0.70 were used for calculating the $t_{1/2}$ using the equation $t_{1/2} = \ln(2)/b$. Standard deviations of the mean and Student's t test were determined using Excel for all experiments, which were repeated at least three times.

Computer-predicted secondary structures

The *mfold* web server (www.bioinfo.rpi.edu/applications/mfold/old/rna/) was used to predict the secondary structure of the DEN2 3′UTR with and without the 3′SL domain using standard conditions (Zuker, 2003).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2004.08.004.

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