

Dengue Virus Utilizes a Novel Strategy for Translation Initiation When Cap-Dependent Translation Is Inhibited

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Viruses have developed numerous mechanisms to usurp the host cell translation apparatus. Dengue virus (DEN) and other flaviviruses, such as West Nile and yellow fever viruses, contain a 5' m⁷GpppN-capped positive-sense RNA genome with a nonpolyadenylated 3' untranslated region (UTR) that has been presumed to undergo translation in a cap-dependent manner. However, the means by which the DEN genome is translated effectively in the presence of capped, polyadenylated cellular mRNAs is unknown. This report demonstrates that DEN replication and translation are not affected under conditions that inhibit cap-dependent translation by targeting the cap-binding protein eukaryotic initiation factor 4E, a key regulator of cellular translation. We further show that under cellular conditions in which translation factors are limiting, DEN can alternate between canonical cap-dependent translation initiation and a noncanonical mechanism that appears not to require a functional m⁷G cap. This DEN noncanonical translation is not mediated by an internal ribosome entry site but requires the interaction of the DEN 5' and 3' UTRs for activity, suggesting a novel strategy for translation of animal viruses.

Protein synthesis consists of an intricate series of events requiring components that are too numerous to be encoded by viral genomes. Therefore, viruses depend on the host cell translation machinery for the production of viral proteins and, as a result, have developed novel mechanisms to compete with cellular mRNAs for limiting translation factors. Specifically, many viral RNAs are able to bypass dependency upon an m⁷GpppN cap structure for translation initiation. During cap-dependent translation initiation, the eukaryotic initiation factor 4E (eIF4E), a component of the cap-binding complex eIF4F, recognizes an m⁷GpppN cap structure at the 5' end of viral and cellular mRNAs. The eIF4F cap-binding complex consists of eIF4E, an adaptor protein (eIF4G), and a helicase complex (eIF4A plus cofactor eIF4B). Only when bound to the cap structure can the eIF4F complex recruit the ribosome complex to the mRNA (25). For animal viruses and a number of eukaryotic mRNAs, cap-independent translation initiation is achieved through the use of an internal ribosome entry site (IRES) (21), examples of which are found in the 5' untranslated region (UTR) of the genomes of picorna- and hepaciviruses (50). Cap-independent translation initiation in plant viruses that lack an IRES structure does exist and involves the use of structures located in the viral 5' or 3' UTR (51). For instance, RNA structures in the 3' UTR of the *Luteovirus* barley yellow dwarf virus interact with eIF4F and presumably deliver them to the viral 5' UTR via long-range base pairing (31, 51), and eIF4F binds to the 3' UTR of the *Tombusvirus* satellite tobacco mosaic virus to facilitate cap-independent translation (23). In addition, RNA-RNA interactions between the 5' and 3' UTRs of certain *Necrovirus* and *Dianthovirus* RNAs also mediate cap-independent translation (47, 49, 57).

Despite the multiple mechanisms of cap-independent translation initiation found in plant virus families, the IRES is the only form of cap-independent translation that has been described for animal viruses.

In response to cellular stresses, such as nutrient deprivation, heat shock, and viral infection, and to normal cellular processes, such as mitosis and differentiation, eukaryotic cells can precisely and reversibly modify the activity of the translation machinery. A key point of control is the availability of eIF4E (26). One important method of regulating eIF4F binding to capped mRNAs is via sequestration of eIF4E from the cap-binding complex by the hypophosphorylated form of the eIF4E-binding proteins (4E-BPs) (25). This interaction, in turn, modulates the level of cap-dependent translation.

During short periods of inhibition of cellular cap-dependent translation, such as apoptosis or entry into mitosis, several cellular transcripts (e.g., ornithine decarboxylase, cellular inhibitors of apoptosis, and p34Cdc2-related protein kinases) have been shown to undergo a switch from cap-dependent to cap-independent translation (10, 54, 60). Similarly, some viral mRNAs have been shown to switch to more efficient forms of translation initiation during certain stages of the viral life cycle or when cellular translation factors are limiting (often due to inactivation by viral proteases). For example, translation from the picornavirus IRES has been shown to be upregulated in certain cell types (6, 18). One cap-dependent mechanism by which viral translation is initiated more efficiently than eukaryotic mRNA is through shunting of the ribosome. Viruses that undergo ribosome shunting (e.g., adenovirus, Sendai virus, and cauliflower mosaic virus) directly translocate the ribosome from the upstream initiation complex to the AUG initiator codon without requiring the eIF4A helicase to unwind RNA secondary structure (33, 43, 63). For adenovirus, the viral 5' UTR directs both the ribosome scanning and shunting mechanisms when eIF4F is abundant but exclusively uses the ribosome shunting mechanism during late adenovirus infection,

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when eIF4F is inactivated (63). Characterization of the specialized circumstances involved in the developmentally regulated translation of multifunctional viral mRNAs will likely reveal important factors that define pathogenesis.

Dengue virus (DEN) is a mosquito-borne human pathogen and a major public health threat worldwide (7). It is an enveloped virus with a positive-sense RNA genome of approximately 10.7 kb with a type I cap structure at the 5' end, a 96-nucleotide (nt) 5' UTR, and a 451-nt nonpolyadenylated 3' UTR (8). DEN and other flaviviruses, such as West Nile, yellow fever, Kunjin, and Japanese encephalitis viruses, are presumed to undergo cap-dependent translation due to the presence of both a 5' cap structure and virally encoded methyltransferase and 5' RNA-triphosphatase activities (2, 20, 62). Despite its presumed dependence on cellular translation factors, DEN has been shown to infect differentiated cells, such as those of the myeloid lineage, which are known to contain limiting amounts of translation factors (29, 41). In view of the fact that DEN does not shut off host cell protein synthesis, the mechanism by which the viral genome competes successfully for cellular translation factors to establish a productive infection is unclear. Here we report that under conditions that inhibit cellular cap-dependent translation, the DEN genome can be translated without a functional cap structure by a novel non-IRES-mediated mechanism that requires both the DEN 5' and 3' UTRs.

MATERIALS AND METHODS

Plaque assay and cell infection. Virus titers of DEN2 strain 16681 (provided by the Centers for Disease Control and Prevention, Fort Collins, CO) in PFU/ml were determined by plaque assay using baby hamster kidney (BHK) 21 clone 15 cells as described previously (16). For infections in the presence of inhibitors of cap-dependent translation, BHK or Vero monkey kidney cells were exposed to viral strains at a multiplicity of infection (MOI) of 1 for 24 h in medium containing either 40 μ M LY294002 (Sigma Chemical Co., St. Louis, MO) or 1 μ M wortmannin (Sigma).

Western blot assay. BHK cells (2×10^5) were exposed to DEN at an MOI of 1 (for visualization of eIF4E, eIF4G, and NS1) or 10 (for 4E-BP1) in the presence of 40 μ M LY294002 or 1 μ M wortmannin for 12 h. Cells were then harvested, lysed in a Triton X-100 solution, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane and visualized by Western blotting with polyclonal anti-4E-BP1 (Cell Signaling Technologies, Beverly, MA), anti-eIF4E (Cell Signaling Technologies), anti-eIF4G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-actin (Santa Cruz Biotechnology, Inc.), or anti-DEN NS1 monoclonal antibodies (P. R. Beatty and E. Harris, unpublished results). Equal amounts of protein (25 μ g) were loaded per lane except for 4E-BP1 blots, where equal numbers of cells were loaded in each lane. Quantitation was performed using a Bio-Rad Chemi-Doc system (Bio-Rad, Hercules, CA).

Infectious clone and reporter constructs. Infectious DEN RNA was transcribed from the pD2/IC infectious clone (gift of Richard Kinney, Centers for Disease Control and Prevention, Fort Collins, CO) from the T7 promoter using the RiboMAX large-scale RNA production system (Promega, Madison, WI) to incorporate either an m⁷GpppA (New England Biolabs, Beverly, MA) or an ApppA (Sigma) as the 5'-terminal nucleotide. RNA reporter constructs used in this study were generated using the pGL3 vector backbone (Promega) and either the 5' UTR of the human β -globin gene or the DEN2 5' UTR sequence fused to the luciferase (Luc) gene, followed by either the DEN2 3' UTR sequence or a 268-nt vector sequence plus a 60-nt polyadenylated [poly(A)] tail (34). As above, the RNA was transcribed from the T7 promoter to contain either an m⁷GpppA, m⁷GpppG, ApppG (New England Biolabs), or an ApppA as the 5'-terminal nucleotide using the RiboMAX large-scale RNA production system. The dicistronic constructs (35) used in the IRES experiments were in vitro transcribed from the T7 promoter using the RiboMAX large-scale RNA production system.

Metabolic labeling of DEN-infected cells. For experiments in which translation of DEN NS5 was measured in comparison to cellular proteins, cells were either

treated with inhibitors during infection or transfected with a small interfering RNA (siRNA)-generating plasmid for 48 h prior to infection. Cells were then exposed to DEN at an MOI of 100 and incubated in RPMI supplemented with 5% fetal bovine serum (FBS) for 12 or 24 h. Prior to harvest, cells were incubated for 1 h in 500 μ l of cysteine- and methionine-deficient RPMI medium with 5% dialyzed FBS. Newly synthesized proteins were labeled with [³⁵S]cysteine-methionine (100 μ Ci) for 30 min. The cells were detached with Hank's balanced salt solution plus 5 mM EDTA, washed twice in cold PBS, and counted, and then equal numbers of cells were resuspended in 25 μ l of SDS sample buffer. Proteins were separated on an 8% SDS-polyacrylamide gel, which was then exposed to a Molecular Dynamics PhosphorImager detection system (Amersham Biosciences, Piscataway, NJ). Proteins were quantitated using ImageQuant image analysis software (Amersham Biosciences).

In vitro translation assay. In vitro translation extracts were generated from BHK cells by a previously published method (19). To compare translation of RNA reporter constructs in the presence of m⁷GpppA or ApppA cap analog competitor, in vitro translation extracts were prepared and then incubated with increasing concentrations (0 to 1.25 mM) of m⁷GpppA cap analog (NEB) or ApppA (Sigma) for 15 min at room temperature. These extracts were then programmed with molar equivalents of each RNA reporter construct transcribed to contain an m⁷GpppN cap structure at its 5' end, as indicated above. The lysates were incubated at 30°C for 60 min, and Luc activity was measured using the luciferase assay reagent (Promega) and a TD20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA transfection. Infectious viral RNA generated from the pD2/IC DEN infectious clone and RNA reporter constructs was transfected into cells using Lipofectamine 2000 (Gibco BRL, Carlsbad, CA). Immediately prior to transfection, cell monolayers were washed with Optimem medium (Gibco BRL). In a polystyrene tube, 1 ml of Optimem was mixed with 5 μ l of transfection reagent and 50 μ l of concentrated viral RNA per well of a 12-well plate. For pD2/IC RNA, the solution was added to each well, and cells were incubated at 37°C for 4 to 6 h. Cells were then washed two times with 2 ml of RPMI plus 10% FBS, 40 μ M LY294002 or 1 μ M wortmannin was added, and cells were further incubated at 37°C for 24 h. For RNA reporter constructs, monolayers were washed 1 h posttransfection, and 40 μ M LY294002 or 1 μ M wortmannin was added. Cells were incubated at 37°C for 8 to 12 h, and Luc activity was assayed by luminometry. For all experiments, efficiency of translation was determined via real-time reverse transcription-PCR (RT-PCR) of RNA extracted from cells when transfection reagent was removed 1 h after its addition to the cells.

siRNA expression. An eIF4E-specific siRNA construct was generated from the pRF42 plasmid backbone (46). An oligonucleotide encoding sequence from the eIF4E gene (nt -9 to +12; GenBank accession no. BC010759) (13) in the sense and the antisense directions separated by a 10-nt hairpin was inserted into the BbsI site of the pRF42 vector. BHK cells were transfected with 4 μ g of either an RNA inhibitory (RNAi) construct targeting eIF4E, a control reverse-sense RNAi construct, or empty vector in the presence of Lipofectamine 2000 liposomal reagent and transfection medium (Invitrogen, Carlsbad, CA). After 2 h, monolayers were washed, cells were incubated at 37°C, and siRNA inhibition at 48 h was determined by quantitation of eIF4E suppression by Western blotting using anti-eIF4E antibodies. An equal number of cells was loaded in each lane.

Dicistronic reporter constructs. The dicistronic constructs used in the IRES experiments were derived from the dicistronic encephalomyocarditis virus (EMCV)-IRES construct and the Δ EMCV-IRES construct (gift of Peter Sarnow, Stanford University) (35). For dicistronic constructs containing DEN sequences, the DEN 5' UTR was inserted between the mutant EMCV IRES and the firefly Luc gene using a PCR-derived fragment (EcoRI-NarI). The 451-nt DEN 3' UTR was inserted between the NarI and the XbaI sites at the 3' end of the firefly Luc gene in the construct containing the DEN 5' UTR.

RESULTS

DEN translation is resistant to suppression of cap-dependent translation. To delineate the ability of DEN to compete with eukaryotic mRNAs for translation factors involved in cap-dependent translation, several strategies were pursued. First, the compounds LY294002 and wortmannin were used to mimic cellular conditions under which eIF4E activity is suppressed. These drugs inhibit the phosphoinositol-3 kinase pathway, leading to hypophosphorylation of 4E-binding protein 1 (4E-BP1), which results in sequestration of the cap-binding protein

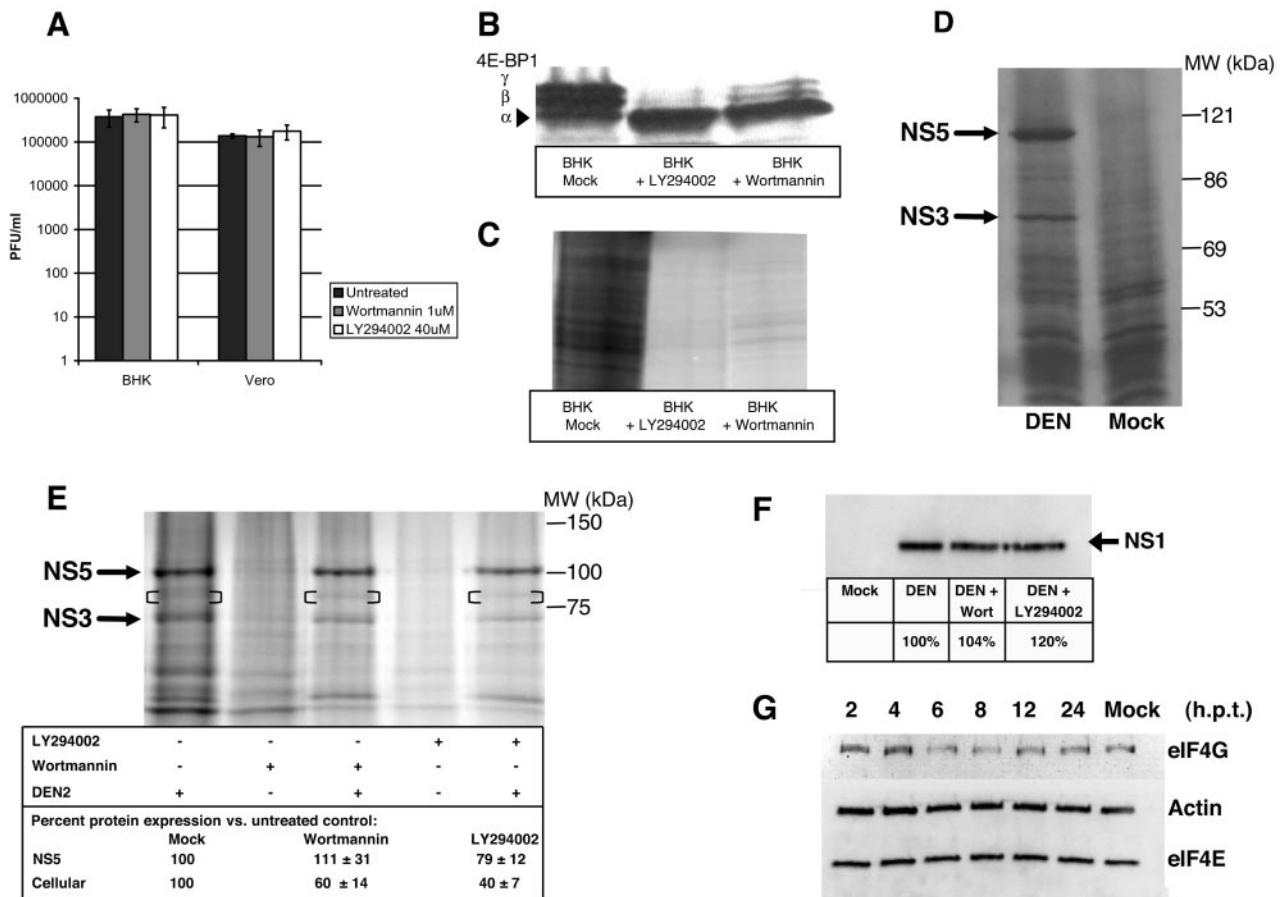


FIG. 1. DEN replication and translation are resistant to inhibitors of cap-dependent translation. (A) DEN replicates in cells exposed to inhibitors for 24 h. BHK or Vero cells (2×10^5) were exposed to DEN2 strain 16681 and simultaneously treated with 40 μ M LY294002 or 1 μ M wortmannin per well or mock treated. Cells were incubated for 24 h at 37°C, and then cell supernatants were collected, and infectious virus titer was determined using BHK21 cells (PFU/ml). The data are expressed as an average of three experiments. Error bars indicate standard errors of the means. (B) Treatment of BHK cells with LY294002 or wortmannin results in hypophosphorylation of 4E-BP1. Cells treated as described above were harvested 24 h postinfection. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and 4E-BP1 was visualized by immunoblotting with polyclonal anti-4E-BP1 antibody. Indicated are the three phosphorylation states of 4E-BP1, α , β , and γ ; α designates the lowest state of phosphorylation. (C) Treatment of BHK cells with LY294002 or wortmannin inhibits cellular protein synthesis. Cells were treated with wortmannin or LY294002 or mock treated as for panel A for 12 h and then metabolically labeled. Cells were starved in cysteine-methionine-free medium 1 h prior to labeling and then pulsed with 150 μ Ci of [35 S]cysteine-methionine for 30 min, harvested, and analyzed by SDS-PAGE. (D) DEN infection does not inhibit cellular translation. To metabolically label total cellular protein in uninfected cells or cells infected with DEN2 for 24 h, BHK cells were starved in cysteine-methionine-free medium and pulsed with [35 S]cysteine-methionine, harvested, and analyzed by SDS-PAGE as for panel C. DEN NS5 and NS3 are indicated by arrows. Data are representative of three experiments. (E) DEN RNA is translated in inhibitor-treated cells. Cells were treated with LY294002 or wortmannin or mock treated as described for panel A and were infected with DEN or mock infected. At 12 h postinfection, total cellular protein was metabolically labeled as described for panel C. Lysates from equal numbers of cells were analyzed by SDS-PAGE. DEN NS5 (arrow) and representative cellular proteins were quantitated as percent protein relative to the untreated control. Brackets indicate cellular proteins that were quantitated. The gel is representative of four experiments, and the average of the four experiments was computed and is presented \pm the standard deviation. (F) Synthesis of DEN NS1 is not reduced in inhibitor-treated cells. Cells treated as for panel E were harvested 12 h postinfection. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and NS1 was visualized by immunoblotting with monoclonal anti-NS1 antibody. Actin was visualized on the same blot, and NS1 levels were normalized to actin. (G) eIF4G and eIF4E levels are not significantly affected by treatment with wortmannin. BHK cells were treated with 1 μ M wortmannin and harvested 4 to 24 h posttreatment. eIF4G and eIF4E were visualized by Western blotting with polyclonal anti-eIF4G antibody or anti-eIF4E antibody, respectively. Actin was included as a loading control. h.p.t., hours posttreatment.

eIF4E from eIF4F and inhibition of cap-dependent translation (61). When BHK cells were incubated with DEN in the presence of LY294002 or wortmannin, equivalent virus titers were observed at 24 h postinfection in treated and mock-treated cells (Fig. 1A). The phosphorylation state of 4E-BP1 in these cells was examined, and the predominant species in the cells treated with LY294002 or wortmannin for 24 h was found to be the hypophos-

phorylated (alpha) form (27), as determined by Western blotting (Fig. 1B). The inhibition of cellular protein synthesis was verified by biosynthetically labeling cells that were either untreated or treated with LY294002 or wortmannin (Fig. 1C). To further confirm these results, the experiments were repeated in Vero cells treated with LY294002 or wortmannin, and similar results were obtained (Fig. 1A). Additionally, DEN propagation in the pres-

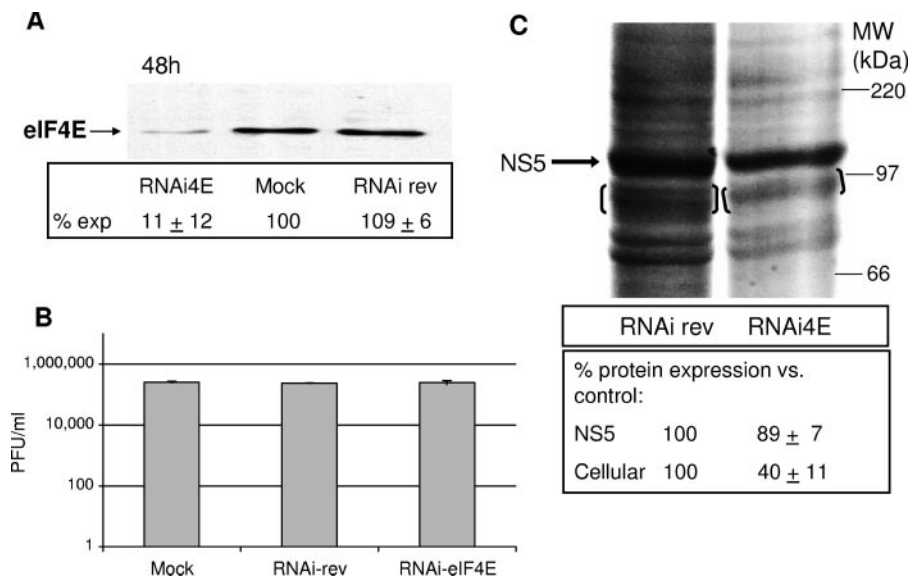


FIG. 2. DEN replication and translation resist siRNA-mediated depletion of eIF4E. **A**. Treatment with an eIF4E-targeted siRNA for 48 h reduces eIF4E expression in cells. Cells were transfected with an RNAi construct targeting eIF4E, a control RNAi construct, or empty vector. After 2 h, monolayers were washed and incubated for 48 h at 37°C. Cell lysates were harvested at 48 h and separated by SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. eIF4E was visualized by Western blotting with polyclonal anti-eIF4E antibody and quantitated. eIF4E is presented as a percentage of protein relative to the empty vector negative control ± the standard deviation from three experiments. **B**. siRNA-mediated suppression of eIF4E does not affect DEN replication after 24 h. Cells described above were exposed to DEN2 strain 16681 at an MOI of 10. After 2 h, monolayers were washed, and the cells were incubated for an additional 24 h at 37°C. Cell supernatants were collected, and titers were determined using BHK cells (PFU/ml). The data are expressed as an average of three experiments. Error bars indicate standard deviations. **C**. Suppression of eIF4E does not affect DEN translation. Cells were transfected with an RNAi construct targeting eIF4E, a control RNAi construct, or empty vector for 48 h preinfection. siRNA-treated cells were exposed to DEN at an MOI of 100 for 12 h. One hour prior to labeling, cells were starved in cysteine-methionine-free medium. Cells were then pulsed with 150 μCi of [³⁵S]cysteine-methionine for 30 min, cell lysates were harvested, and proteins were separated by SDS-PAGE. Cellular and viral NS5 (arrow) proteins were analyzed, and data are presented as described for Fig. 1E. Brackets indicate cellular proteins that were quantitated.

ence of rapamycin, which acts through the alternative mammalian target of rapamycin pathway to dephosphorylate 4E-BP1, again resulted in equal amounts of viral progeny as in mock-treated cells (data not shown).

Cellular infection with the majority of animal viruses results in the inhibition of host protein synthesis through a variety of mechanisms, including cleavage of certain translation factors, modulation of the ratio of cellular message to viral RNA levels, or prevention of the export of cellular messages from the nucleus (55, 58). However, when metabolically labeled total cellular proteins from uninfected BHK cells or those infected with DEN for 24 h were analyzed, DEN infection appeared to exert no effect on cellular protein synthesis (Fig. 1D), consistent with earlier reports of other flaviviruses (4). The identity of DEN protein NS5 was previously confirmed by Western blotting (17) (data not shown). To determine the ability of DEN to translate in the presence of wortmannin and LY294002, total cellular proteins from DEN-infected cells were metabolically labeled and examined. Treatment with wortmannin (Fig. 1E, lanes 2 and 3) or LY294002 (Fig. 1E, lanes 4 and 5) suppressed cellular protein synthesis equivalently, regardless of infection status. In these experiments, in contrast to the approximately 50% reduction of cellular protein synthesis resulting from LY294002 or wortmannin treatment, synthesis of DEN proteins, represented by NS5, was less affected (0 to 20% reduction) (Fig. 1D). Furthermore, quantitation of DEN NS1 in treated versus untreated cells by Western blotting revealed uniform levels of protein ex-

pression (Fig. 1F). Levels of eIF4F components remained stable in cells over the course of treatment (Fig. 1G). Likewise, translation of DEN NS5 was relatively unaffected in cells depleted of eIF4E through the overexpression of a nonphosphorylatable form of 4E-BP1 (44), whereas translation of cellular proteins was reduced (data not shown). Together, these results indicate that, despite the presence of a 5' cap structure on the DEN genome, DEN replication and translation are resistant to inhibition of cap-dependent translation.

As an alternative and more specific approach to inhibit cap-dependent translation, siRNA-mediated gene silencing was employed to suppress expression of the prototypical form of mammalian eIF4E, eIF4E-1 (38). A plasmid encoding a 21-nt hairpin targeting a sequence at the 5' end of eIF4E-1 (13, 46), a control plasmid containing the eIF4E-1 sequence in reverse, and a control empty vector were transfected into BHK cells. Expression of the eIF4E-1-specific siRNA reduced levels of eIF4E-1 by approximately 90% in comparison with the control plasmid, as determined by Western analysis 48 h after transfection (Fig. 2A). Cells were infected with DEN at 48 h posttransfection, and the virus titers were measured at 24 h postinfection. Similar to results obtained with LY294002 and wortmannin (Fig. 1A), suppression of eIF4E-1 via siRNA had no effect on the ability of DEN to replicate (Fig. 2B). Also consistent with the LY294002 and wortmannin results (Fig. 1E), total cellular protein synthesis was decreased by 60% in the eIF4E-1-depleted cells in comparison with cells containing the control siRNA plasmids, whereas DEN NS5

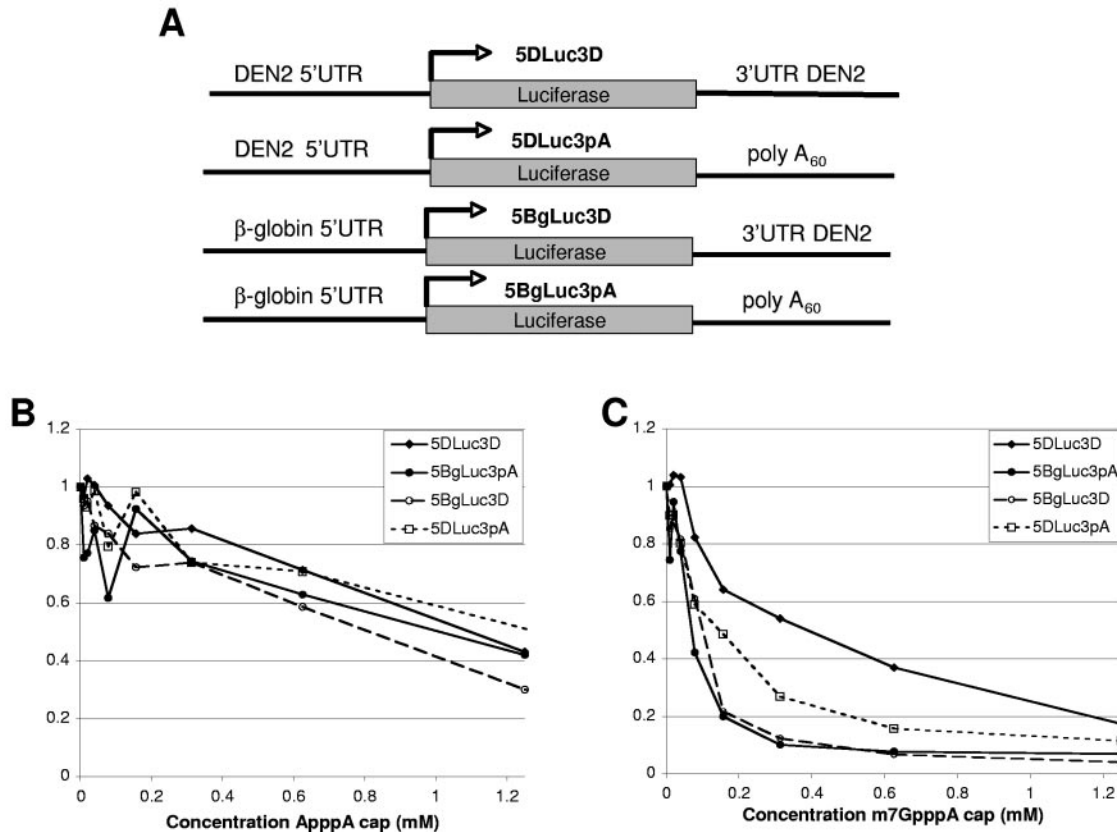


FIG. 3. Translation of DEN reporter RNAs is resistant to competition for eIF4E in vitro. (A) Schematic diagram of RNA reporter constructs. RNA reporter constructs contain either the DEN2 5' UTR or the human β -globin (β g) 5' UTR fused to the firefly Luc gene, followed by either the DEN2 3' UTR or a vector sequence plus a 60-mer poly(A) tail. RNA transcripts were generated from a T7 promoter. (B) ApppA cap analog does not differentially affect translation of DEN reporter constructs. In vitro translation extracts were incubated with increasing concentrations of ApppA cap analog for 15 min at room temperature and then programmed with molar equivalents of RNA reporter constructs, as indicated. Luc activity was measured after 1 h and is presented as a function of cap concentration. Data shown are representative of four experiments. (C) DEN translation resists competition for eIF4E. In vitro translation extracts were incubated with increasing concentrations of m⁷GpppA cap analog for 15 min at room temperature and then programmed with molar equivalents of RNA reporter constructs, as indicated. Luc activity was measured after 1 h and is presented as a function of cap concentration. Data shown are representative of six experiments.

protein expression was only decreased by $\sim 10\%$ (Fig. 2C). These results confirm that DEN translation can occur when levels of eIF4E are reduced.

DEN translates independently of the cap structure under conditions of reduced eIF4E. The resistance of DEN to inhibition of eIF4E activity suggests that DEN may be capable of cap-independent translation. To determine more directly the effects of eIF4E sequestration on DEN translation, a series of reporter constructs was generated containing either the DEN 5' UTR or the human β -globin 5' UTR fused to the firefly luciferase (Luc) gene, followed by either the DEN 3' UTR or a 268-nt vector sequence that ends with a 60-mer poly(A) tail (Fig. 3A). With these constructs, competition assays were performed using an m⁷GpppA cap analog, which binds to the cap-binding pocket of eIF4E, removing it from the pool of functional translation factors. Thus, this treatment should suppress cap-dependent translation in a dose-dependent manner. In addition, the same competition was performed with ApppA cap analog to control for specificity and Mg²⁺-chelating properties of the cap compounds. In vitro translation extracts prepared from BHK cells were incubated with increasing concen-

trations of either m⁷GpppA or ApppA cap analog for 15 min and then programmed with molar equivalents of each m⁷GpppN-capped RNA reporter construct. As expected, in the control ApppA experiments, translation of all four constructs was equally affected in a nonspecific manner by increasing doses of cap analog (Fig. 3B). In contrast, at increasing concentrations of m⁷GpppA cap analog, translation of the construct containing both the DEN 5' and 3' UTRs exhibited less sensitivity to the sequestration of eIF4E than the other constructs (Fig. 3C). Compared with the 5DLuc3D construct, translation from the constructs containing the β -globin 5' UTR was suppressed 80% at approximately eightfold-lower concentrations of cap analog, irrespective of the sequence of the 3' UTR. The contribution of the DEN 5' UTR to this effect is evidenced by the intermediate phenotype of the construct containing the DEN 5' UTR and a poly(A) tail. These results demonstrate that DEN translation, mediated by the presence of both the 5' and 3' UTRs, has a reduced dependence on eIF4E.

To directly assess the influence of a functional 5' m⁷GpppN cap structure on DEN translation, the reporter constructs described above were transcribed in vitro to contain

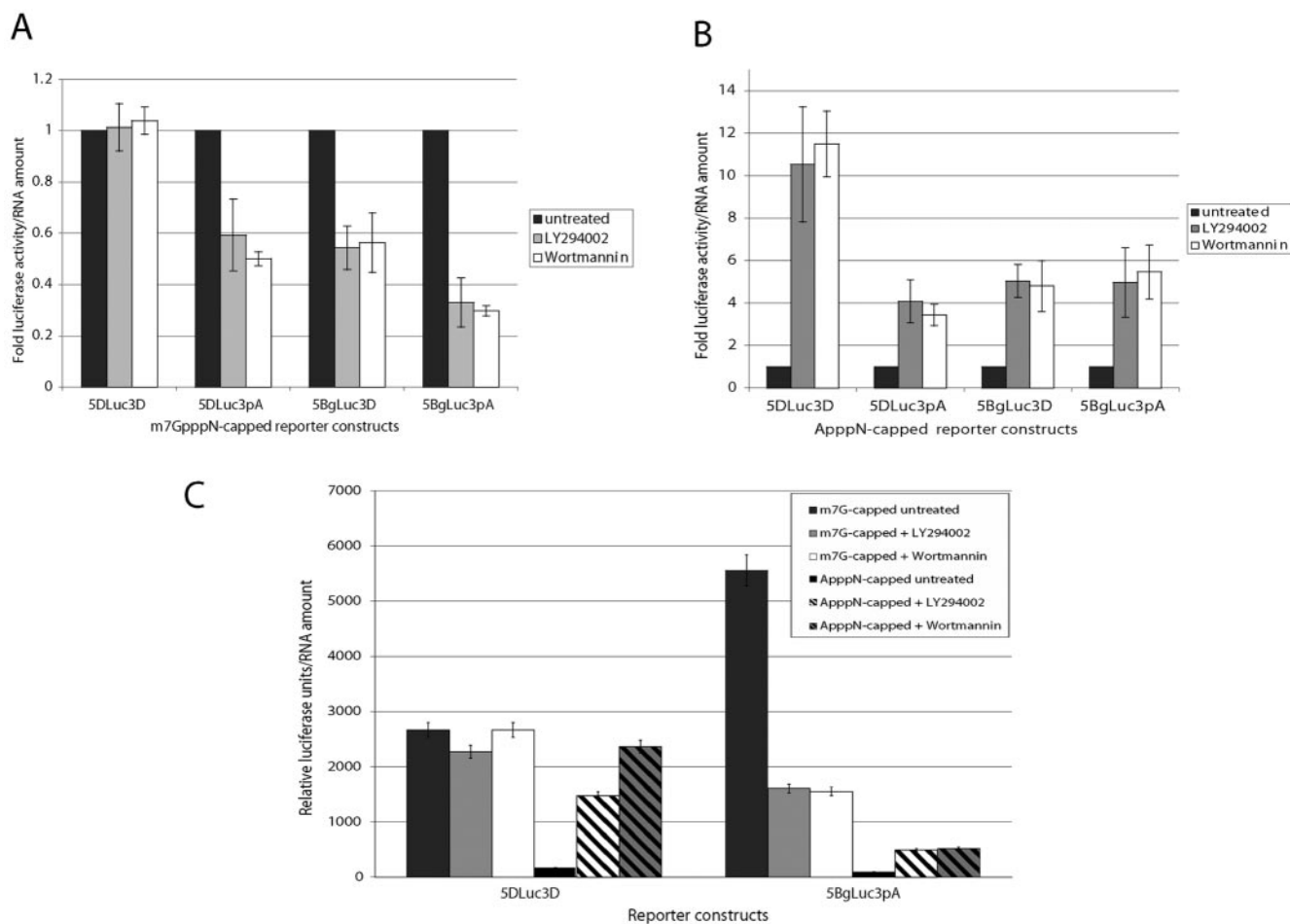


FIG. 4. Translation of DEN reporter constructs is resistant to inhibition of cap-dependent translation. (A) The m⁷G-capped DEN reporter construct is translated in inhibitor-treated cells. Cells were transfected with equal amounts of the m⁷GpppN-capped RNA reporter constructs described for Fig. 3A. After 1 h, monolayers were washed, 40 μM LY294002 or 1 μM wortmannin was added, and the cells were incubated at 37°C for 12 h. Luc activity, assayed after 12 h, was normalized to the amount of RNA 1 h posttransfection and then to constructs translated in untreated cells. The data are expressed as an average of four experiments. Error bars indicate standard deviations. (B) The nonfunctionally (ApppA-) capped DEN reporter construct is translated in inhibitor-treated cells. Cells were treated as for panel A, except that the ApppN-capped RNA reporter constructs were examined. The data are expressed as an average of three experiments. Error bars indicate standard deviations. (C) Translation from ApppA-capped DEN RNA reporter constructs in inhibitor-treated cells is similar to that of m⁷GpppA-capped DEN constructs. Cells were treated as for panels A and B. The data are shown in relative luciferase units and are representative of three experiments. Error bars indicate standard deviations.

either a functional m⁷GpppN cap structure or a nonfunctional 5' ApppN cap, which stabilizes the RNA but is unable to bind eIF4E. The m⁷GpppN- and ApppN-capped RNA transcripts were then translated in BHK cells in the presence of LY294002 or wortmannin. The different RNA constructs have been shown by Northern blot analysis to have similar stability in transfected cells (34); likewise, the stability of the RNAs was unaffected by treatment with the inhibitors, as determined by real-time RT-PCR analysis (±5% of untreated control). When Luc activity was measured 12 h posttransfection of m⁷GpppN-capped RNAs, only the construct containing both the DEN 5' and 3' UTRs was fully resistant to the effects of the drugs, whereas translation of all other constructs was reduced by at least 50% (Fig. 4A). Translation from a control reporter construct containing the 5' UTR of the human actin gene was similarly reduced in the presence of translational inhibitors (data not shown). When ApppN-capped RNAs were examined, transla-

tion of the four constructs in untreated cells was equally inefficient but was slightly improved in cells treated with inhibitors (Fig. 4B), presumably due to the increased availability of eIF4G (3, 59). However, in the presence of inhibitors of cap-dependent translation, Luc activity from only the ApppN-capped construct containing both the DEN 5' and 3' UTRs was substantially increased (Fig. 4B) to levels similar to that of the m⁷GpppN-capped DEN construct (Fig. 4C). These results suggest that, contrary to prior belief (45), the capped DEN genome can be translated independently of the cap structure under conditions that inhibit translation of cap-dependent cellular messages. Moreover, both the DEN 5' and 3' UTRs are necessary to mediate this effect. As the DEN genome likely contains an m⁷G cap throughout its infectious life cycle, we will refer to the alternative mechanism of DEN translation that can function in the absence of an m⁷G cap as noncanonical DEN translation.

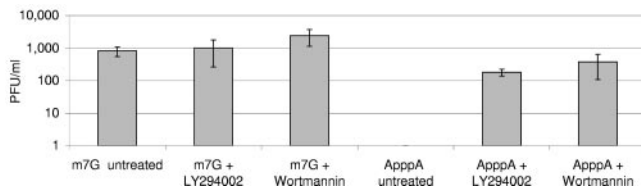


FIG. 5. DEN alternates between canonical cap-dependent and noncanonical translation. Equal amounts of functionally (m^7GpppA) or nonfunctionally (ApppA) capped *in vitro* transcripts generated from the DEN2 infectious clone were transfected into cells for 4 h. Cells were washed and incubated with LY294002 or wortmannin for 24 h. Supernatants were then collected, and titers were determined by plaque assay. The data are expressed as an average of three experiments. Error bars indicate standard deviations.

The DEN genome can switch from canonical cap-dependent translation to a noncanonical translation initiation mechanism. The observation that noncanonical DEN translation is upregulated from ApppA-capped DEN reporter constructs under conditions of reduced eIF4E (Fig. 4B and C) led us to examine the ability of the DEN genome in its entirety to replicate in the absence of a functional cap structure. In this experiment, RNA was transcribed from the DEN2 infectious clone to contain either a functional 5' m^7GpppA cap structure or a nonfunctional 5' ApppA cap and was transfected into untreated or inhibitor-treated cells. Equal amounts of the m^7GpppA -capped and the ApppA-capped DEN2 RNAs were observed by real-time RT-PCR at both 1 h and 12 h posttransfection (data not shown). When supernatants of untreated cells were analyzed by plaque assay 24 h posttransfection, no infectious progeny were observed following transfection of the ApppA-capped viral RNA, compared with titers of 10^3 PFU/ml obtained following transfection of m^7GpppA -capped viral RNA (Fig. 5, columns 1 and 4), indicating that DEN translates exclusively via its 5' m^7GpppA cap structure in cells competent for cap-dependent translation. Consistent with earlier observations (Fig. 1A), viral titers from cells transfected with the m^7GpppA -capped viral RNAs were unaffected by the presence of inhibitors of cap-dependent translation (Fig. 5, columns 2 and 3). Importantly, as predicted by the previous results obtained with reporter RNAs, viral titers from inhibitor-treated cells transfected with the nonfunctionally capped viral genomes were increased from 0 PFU to 20 to 30% of that obtained following transfection of the m^7GpppA -capped genome (Fig. 5, columns 5 and 6). This indicates that a productive DEN infection can be initiated independently of the cap structure under conditions of reduced eIF4E.

DEN does not contain an IRES element. The discovery of the ability of DEN to translate and replicate independently of a cap structure led us to investigate the existence of IRES activity in the DEN UTRs. A series of dicistronic reporter constructs (35) were generated that encode a cap-dependent 5' UTR fused to the *Renilla* luciferase gene, followed by a mutated EMCV IRES to prevent read-through (36). The second cistron consists of the DEN 5' UTR, a functional EMCV IRES (positive control), or the mutated EMCV IRES (negative control) fused to the firefly luciferase gene, and finally the DEN 3' UTR or a 60-mer poly(A) tail (Fig. 6A). If DEN RNA contains IRES activity, firefly Luc should be expressed from the dicis-

tronic construct containing the internal (uncapped) DEN 5' UTR. When *in vitro*-transcribed RNAs derived from the dicistronic constructs were transfected into cells, cap-dependent *Renilla* Luc activity was equivalent for all constructs (Fig. 6B). However, IRES activity was apparent only with the construct containing the positive control (Fig. 6C). Neither the presence of the DEN 3' UTR nor the addition of inhibitors of cap-dependent translation initiation altered these results (data not shown), indicating that noncanonical DEN translation is not mediated by internal ribosome entry.

DISCUSSION

We describe a situation wherein the flavivirus DEN appears to have evolved a novel mechanism to effectively recruit translation initiation factors under conditions in which these factors are limiting within the cell. We show that treatment of cells with inhibitors of cellular cap-dependent translation (e.g., LY294002, wortmannin, or eIF4E-specific siRNAs) leads to inhibition of cellular translation but does not affect the yield of infectious DEN viral progeny, the level of DEN protein synthesis, or the translation of RNA reporter constructs containing both the DEN 5' and the 3' UTRs. Experiments in which increasing concentrations of m^7G cap analog were used to deplete eIF4E in translation extracts indicated that, in comparison to cellular 5' leaders, translation of reporter constructs containing both the DEN 5' and 3' UTRs is resistant to competition for eIF4E. These results suggest that the presence and/or interaction of both the DEN 5' UTR and the DEN 3' UTR is necessary to mediate resistance to the effects of eIF4E suppression. Furthermore, although the nonfunctionally capped viral genome is unable to replicate independently of an m^7G cap structure under normal cellular conditions, when eIF4E is inhibited, replication of the ApppA-capped DEN infectious clone is upregulated. However, despite its resistance to inhibitors of cap-dependent translation, we find that the DEN UTRs do not contain IRES activity. In summary, our results support a model in which the interaction of the DEN 5' and 3' UTRs allows the virus to translate in either a canonical cap-dependent or a noncanonical alternative manner in response to cellular conditions.

Viruses that undergo cap-dependent translation have evolved a variety of mechanisms through which to compete for components of eIF4F. For example, adenovirus and cauliflower mosaic virus initiate translation via a cap-dependent process called ribosome shunting, whereby the ribosome is directly translocated from the upstream initiation complex to the AUG initiator codon without requiring the eIF4A helicase to unwind RNA secondary structure (33, 64). Similarly, the capped vesicular stomatitis virus (VSV) mRNAs, which are translated under conditions of virally induced suppression of eIF4F, have adapted to this condition by encoding very short 5' leaders, and three out of five VSV mRNA 5' UTRs are less than 14 nt long (9). Alternatively, certain viruses compete for the limiting amounts of translation initiation factors within the cell through the use of viral proteins bound to the 5' or 3' ends of the genome that are able to recruit initiation factors more effectively than other capped mRNAs (12). For example, like the DEN genome, RNA elements of the alfalfa mosaic virus (AMV) and rotavirus genomes possess a 5'-terminal cap struc-

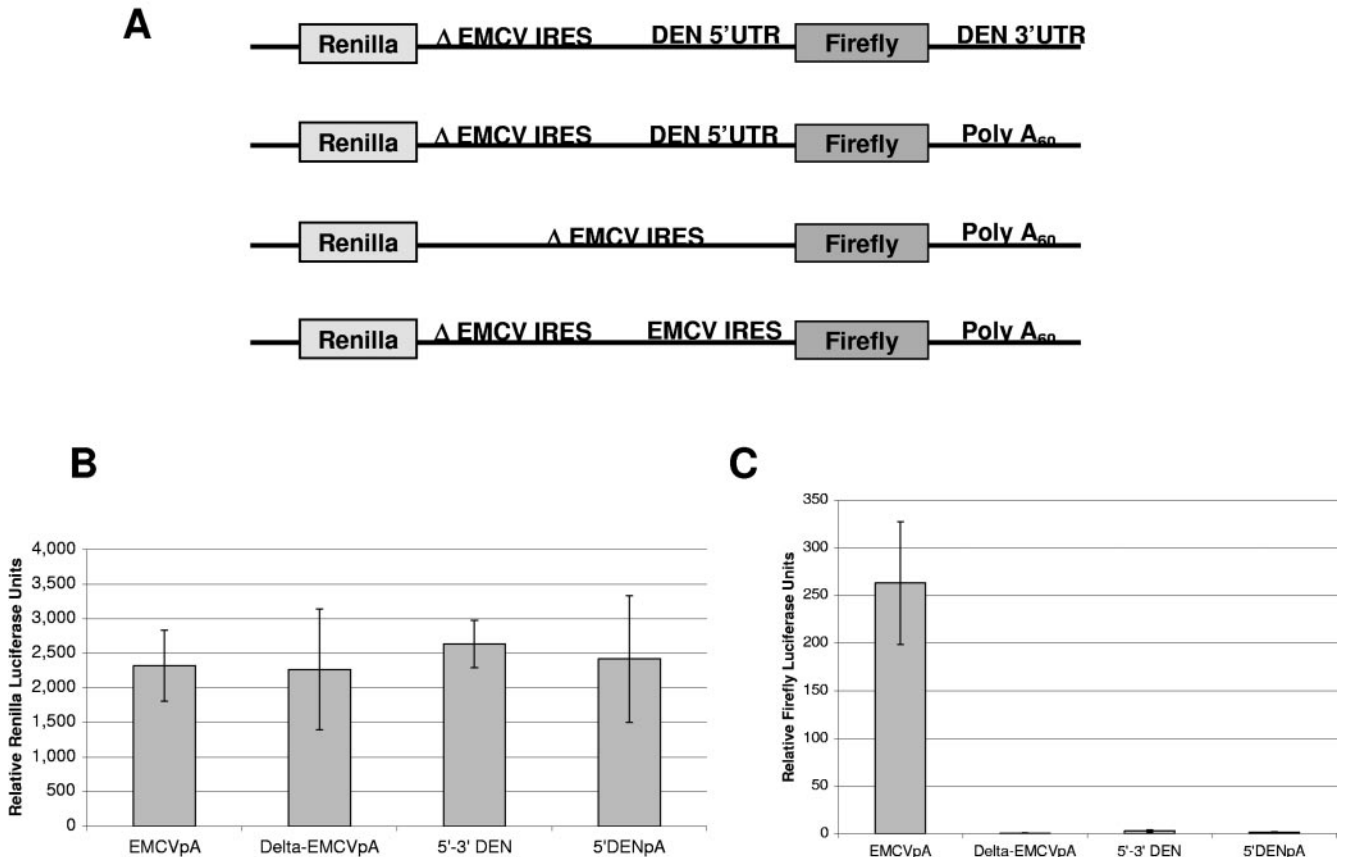


FIG. 6. The DEN 5' and 3' UTRs do not confer IRES activity. A. Dicistronic reporter constructs contain a cap-dependent 5' UTR fused to the *Renilla* Luc gene followed by a mutated (Δ EMCV) IRES and then the DEN 5' UTR or a wild-type EMCV IRES upstream of the firefly Luc gene. The constructs terminate with either the 451-nt DEN2 3' UTR or a 60-mer poly(A) tail. B. Translation of the cap-dependent *Renilla* Luc is equivalent for all constructs. C. DEN 5' and 3' UTRs do not support IRES activity. Cells were transfected with equal amounts of RNA from the dicistronic construct described above. After 1 h, monolayers were washed and 40 μ M LY294002 or 1 μ M wortmannin was added per well. *Renilla* and firefly Luc activities were measured at 12 h. Data are an average of four experiments. Error bars indicate standard deviations.

ture but lack 3'-terminal poly(A) tails. In the case of AMV, the viral coat protein (CP) binds the 3' end of AMV RNAs and mimics the function of the poly(A)-binding protein in translation of cellular mRNAs by interacting with eIF4G to bring about efficient translation of the viral RNAs (40). Similarly, the 3'-end sequences of the rotavirus genome bind the rotavirus NSP3 protein, which both confers stability to the viral mRNAs (15) and interacts with eIF4G with extremely high affinity, thus recruiting it away from cellular messages (53). Furthermore, the influenza virus NS1 protein binds both the influenza virus 5' UTR and eIF4G, directly recruiting the 43S ribosomal complex to the viral 5' terminus (1). Finally, the 5' end of the calicivirus genome is covalently linked to the viral protein VPg, which was found to interact directly with the translation initiation factors eIF3 and eIF4E, acting as a cap substitute and promoting translation from Vpg-linked viral RNA while inhibiting the translation of capped mRNAs (12, 28).

In contrast to the majority of capped mRNAs, we show that the DEN genome, under conditions of reduced eIF4E, can be induced to translate independently of a cap structure. That the DEN UTRs failed to initiate translation via IRES activity implies that initiation of DEN translation may be end dependent. Experiments were performed to test a reporter construct

containing a stable hairpin immediately downstream from the 5' end of the DEN 5' UTR, which should inhibit end-dependent translation initiation by blocking ribosome entry (14, 31, 39). A dramatic decrease in translation of the DEN hairpin-containing construct was observed in untreated as well as eIF4E-depleted *in vitro* translation extracts that support non-canonical DEN translation (S. Paranjape, K. Holden, M. Lee, and E. Harris, unpublished results). These results are consistent with a requirement for a free 5' end for both canonical and non-canonical DEN translation. Such a requirement is not unprecedented; cap-independent translation of members of the plant *Luteovirus* family requires the interaction of the viral 5' and 3' UTRs, which facilitates recruitment of the ribosome to the 5' end of the genome (31, 51). The absence of an IRES in the DEN 5' UTR combined with the requirement for the DEN 3' UTR for cap-independent translation suggest a similar type of communication between the viral UTRs and argue against ribosome shunting.

Although DEN is able to be translated under conditions of low levels of eIF4E, we have obtained data suggesting that DEN requires intact eIF4G for full activity. In extracts treated with the coxsackie B virus 2A protease, which cleaves eIF4G, DEN translation is reduced by 75%, as is the β -globin control

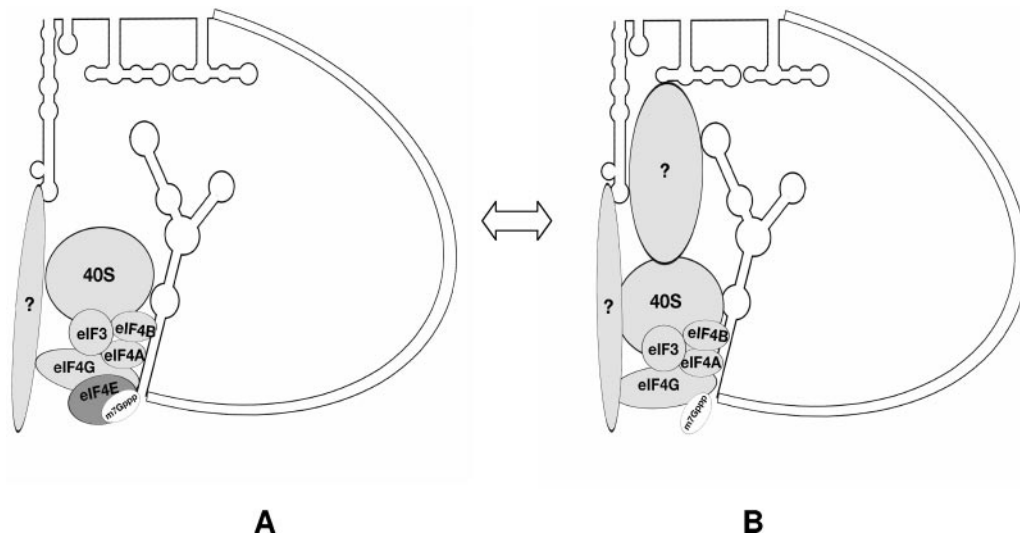


FIG. 7. Model of 5'-3' interactions in canonical (cap-dependent) and noncanonical DEN translation initiation. When eIF4E is abundant, a cap-dependent scanning mechanism of translation initiation occurs (A). When eIF4E is limiting, the DEN 3' UTR interacts with host proteins to deliver and/or stabilize key translation initiation factors at the 5' UTR (B).

construct (data not shown). This is consistent with reduction of Kunjin virus translation by $\sim 75\%$ upon coinfection with poliovirus (56). While the N terminus of eIF4G that is removed by 2A protease includes an eIF4E binding site, the requirement for this fragment for efficient DEN translation may not suggest that eIF4E is necessary but rather may indicate the importance of other binding sites, such as the previously identified PABP binding site or potentially additional binding sites for proteins that are involved in translational enhancement. Alternatively, the N-terminal segment of eIF4G may be necessary for maintaining the proper conformation of eIF4G (30). Additionally, to determine the intrinsic affinity of DEN mRNA for eIF4F relative to an "average" cellular mRNA, filter binding assays and surface plasmon resonance experiments are under way.

We propose a model in which the DEN genome is able to alternate from standard cap-dependent translation to a form of noncanonical translation initiation under conditions of reduced eIF4E (Fig. 7). In this model, in the presence of eIF4E, the DEN 3' UTR functionally replaces a poly(A) tail to enhance translation efficiency via a canonical cap-dependent scanning mechanism (34). However, a decrease in the concentration of eIF4E (or possibly other cellular translation initiation factors) prompts the reorganization of the viral RNP complexes bridging the DEN 5' and 3' UTRs. In this conformation, RNA structures or sequences in the 3' UTR deliver or stabilize translation initiation factors at the 5' end of the RNA, allowing factors such as eIF4G and eIF4A to be recruited while bypassing the requirement for eIF4E. Several proteins have been reported to associate with the DEN 3' UTR, including eEF1A, La, PTB, YB-1, and hnRNP A1 and Q (5, 22, 32) (S. M. Paranjape and E. Harris, unpublished data). These are candidates for proteins that bridge the DEN 5' and 3' UTRs to enhance canonical and/or noncanonical DEN translation.

A likely trigger for noncanonical DEN translation is a reduction in eIF4E levels, due to the critical role of eIF4E in regulation of protein synthesis (26) and as suggested by our

results. The biological relevance of the reduced dependence of DEN translation on eIF4E may correlate with the ability of DEN to translate under conditions in which cellular translation is limited, for example, in differentiated cells such as myeloid and dendritic cells, known to be *in vivo* targets of DEN, that contain lower levels of available eIF4E (29, 41). Consistent with this hypothesis, we have previously shown that an increase in DEN replication coincides with differentiation in human myeloid cells (42), and we are currently evaluating whether translation of DEN proteins and functionally and nonfunctionally capped DEN RNA constructs also increases under these conditions. In addition, mammalian cellular stress response and immune functions, such as the interferon antiviral response (24), may compel viral translation by one mechanism over the other. Finally, noncanonical DEN translation may be elicited only in certain host target cells by the presence of cell- or tissue-specific factors. For example, cap-independent translation of the picornavirus genome has been observed to be specifically regulated in neuronal cells (6, 18). Similarly, cap-independent translation initiation from certain cellular genes has been shown to be dependent upon the presence or activity of specific factors for expression (11, 37, 48, 52).

The existence of two mechanisms by which the DEN genome may be translated is of potential significance for many viruses, such as flaviviruses, that maintain complex life cycles in disparate hosts (i.e., mosquito and human), as well as in multiple cell types within the same host. We find that DEN can switch between cap-dependent translation initiation and a non-IRES-mediated form of noncanonical translation initiation that requires the presence of the 5' and 3' UTRs. Further characterization of this noncanonical mechanism of DEN translation should reveal essential components of the viral life cycle. Ultimately, the report of a capped viral RNA that can alternate between mechanisms of translation in response to cellular environment has implications for cellular tropism, viral transmission, vector and host competence, and antiviral strategies.

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