

## Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3' stem–loop structure

Katherine Lynn Holden <sup>a</sup>, David A. Stein <sup>b</sup>, Theodore C. Pierson <sup>c,1</sup>, Asim A. Ahmed <sup>c</sup>,  
Karen Clyde <sup>a</sup>, Patrick L. Iversen <sup>b</sup>, Eva Harris <sup>a,\*</sup>

<sup>a</sup> Division of Infectious Diseases, School of Public Health, University of California at Berkeley, 140 Warren Hall, Berkeley, CA 94720-7360, USA

<sup>b</sup> AVI BioPharma Inc., 4575 SW Research Way, Corvallis, OR 97333, USA

<sup>c</sup> Department of Microbiology, University of Pennsylvania, 225 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104, USA

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### Abstract

Dengue virus (DEN) is a major public health problem worldwide and causes a spectrum of diseases, for which no antiviral treatments exist. Peptide-conjugated phosphorodiamidate morpholino oligomers (P-PMOs) complementary to the DEN 5' stem–loop (5'SL) and to the DEN 3' cyclization sequence (3'CS) inhibit DEN replication, presumably by blocking critical RNA–RNA or RNA–protein interactions involved in viral translation and/or RNA synthesis. Here, a third P-PMO, complementary to the top of the 3' stem–loop (3'SLT), inhibited DEN replication in BHK cells. Using a novel DEN2 reporter replicon and a DEN2 reporter mRNA, we determined that the 5'SL P-PMO inhibited viral translation, the 3'CS P-PMO blocked viral RNA synthesis but not viral translation, and the 3'SLT P-PMO inhibited both viral translation and RNA synthesis. These results show that the 3'CS and the 3'SL domains regulate DEN translation and RNA synthesis and further demonstrate that P-PMOs are potentially useful as antiviral agents.

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### Introduction

Dengue virus (DEN) causes a spectrum of diseases, from a self-limited febrile illness (dengue fever) to a potentially fatal disease (dengue hemorrhagic fever/dengue shock syndrome). DEN is transmitted to humans primarily by *Aedes aegypti* and *Ae. albopictus* mosquitoes in tropical and sub-tropical regions of the world, exposing 40% of the world's population to potential infection (World Health Organization, 1997). DEN exists as four serotypes (DEN1–4), of which DEN4 is both the progenitor and the most distantly related to the other serotypes.

DEN, a positive-strand RNA virus, is a member of the *Flavivirus* genus within the *Flaviviridae* family. Other flaviviruses include West Nile virus (WNV), yellow fever

virus (YF), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVE), and tick-borne encephalitis virus (TBE) (Westaway et al., 1985), all of which are of major concern for human health. Flavivirus RNA genomes are approximately 11 kilobases in length and have a type I 5' cap structure but are not polyadenylated at the 3' end (Cleaves and Dubin, 1979; Wengler et al., 1978). Upon entry into a target cell and release of the viral RNA from the nucleocapsid, the genome is translated from a single open reading frame (ORF), presumably via a cap-dependent scanning mechanism of translation initiation (Ruiz-Linares et al., 1989). The viral polypeptide is then co- and post-translationally cleaved into three structural proteins (capsid [C], pre-membrane [prM], and envelope [E]) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Hahn et al., 1988). Non-structural proteins replicate the viral RNA via a full-length negative-sense RNA intermediate (Chu and Westaway, 1985; Cleaves et al., 1981; Khromykh et al., 2000). The flaviviral 5'- and 3'-untranslated regions (UTRs) contain several conserved sequences and secondary

\* Corresponding author. Fax: +1 510 642 6350.

E-mail address: [eharris@berkeley.edu](mailto:eharris@berkeley.edu) (E. Harris).

<sup>1</sup> Current address: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 4 Center Drive, Room 216 Bethesda, MD 20892, USA.

structures, some of which are involved in regulating viral translation and RNA synthesis (Markoff, 2003).

In the coding region of C is a conserved sequence of 8 nucleotides (nt) that is complementary to a sequence in the

3'UTR. These are referred to respectively as the 5' cyclization sequence (5'CS) and the 3' cyclization sequence (3'CS) because they are thought to base pair and assist in the circularization of the viral RNA (Hahn et al., 1987). Recently,

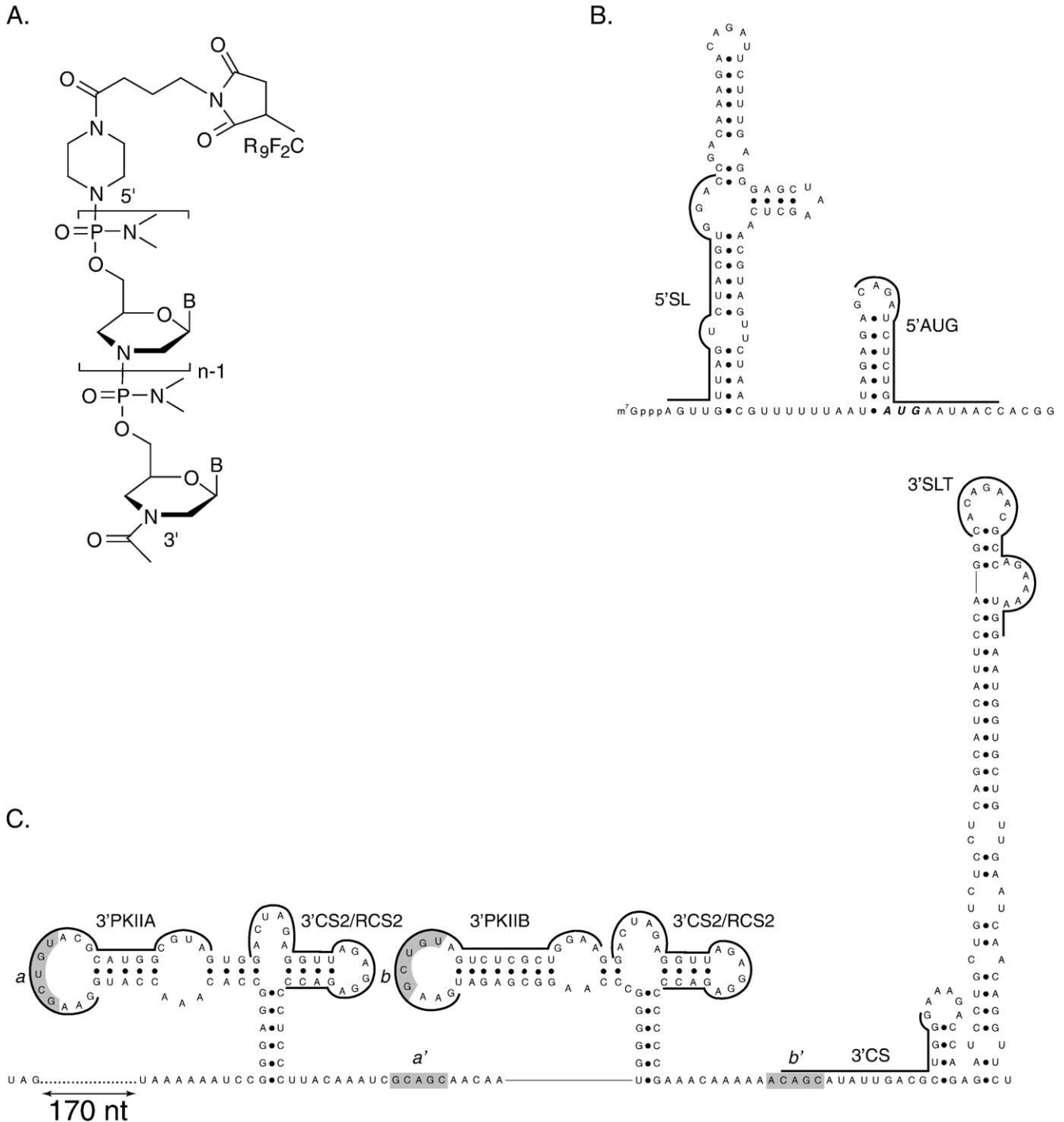


Fig. 1. Schematic diagram of the structure of the P-PMO and the targeted locations in the DEN 5'- and 3'-UTRs. (A) Schematic diagram of the structure of the P-PMO. Arg-rich peptide (R<sub>9</sub>F<sub>2</sub>C) is conjugated to the 5' end of the P-PMO structure. Panel B represents nucleotide bases A, G, C, or T. (B) Locations of P-PMO target sequences in the DEN2 5'UTR. Indicated by lines are target sequences for the DEN 5'SL P-PMO and the 5'AUG P-PMO. The predicted secondary structure of the DEN2 5'UTR was determined using the *mfold* web server (Zuker, 2003). (C) Locations of P-PMO target sequences in the DEN 3'UTR. Indicated by lines adjacent to the 3'UTR sequence are target sequences for the DEN 3'PKIIA, 3'PKIIB, 3'CS2/RCS2, 3'CS, and 3'SLT P-PMOs. The shaded regions indicate the interactions involved in forming the PKIIA and PKIIB pseudoknot secondary structures; the shaded loop sequence is predicted to interact with the shaded region downstream. The secondary structures of the DEN2 3'UTR are based on computer predicted secondary structures (Hahn et al., 1987; Mohan and Padmanabhan, 1991; Olsthoorn and Bol, 2001; Proutski et al., 1999; Shi et al., 1996) and chemical probing of the terminal 100 nt (Shi et al., 1996).

another set of complementary sequences in the DEN 5'- and 3'-UTRs, named the 5' and 3' upstream AUG regions (UARs), were shown to be necessary for the circularization of the genome and for viral replication (Alvarez et al., 2005). The interaction of the 5'- and 3'-CS is critical for flavivirus RNA synthesis (You and Padmanabhan, 1999; Khromykh et al., 2001). The 3'CS is part of a sequence element (CS1) of 23 nucleotides in the 3'UTR that is conserved across all DEN serotypes (Hahn et al., 1987). In the flavivirus 3'UTR, there is a second conserved sequence (CS2) that may be repeated (RCS2) (Hahn et al., 1987). The CS2 and RCS2 of WNV have been shown to play a role in the regulation of viral RNA synthesis (Lo et al., 2003). Some flaviviruses, namely JEV, WNV, and MVE, contain a third conserved sequence (CS3), which is repeated (RCS3) (Hahn et al., 1987).

The flavivirus 3'UTR also contains several conserved secondary structures, including pseudoknots (PKIIA and PKIIB) located upstream of the 3'CS (Olsthoorn and Bol, 2001) and a terminal stem-loop (3'SL) domain immediately downstream of the 3'CS (Brinton et al., 1986; Hahn et al., 1987; Mohan and Padmanabhan, 1991). The DEN2 3'SL domain is critical for viral RNA synthesis (You and Padmanabhan, 1999; Zeng et al., 1998) and enhances translation (Holden and Harris, 2004). In addition, the 3'SL contains a pentanucleotide loop (5'-CACAG-3'), which is highly conserved among the flaviviruses (Wengler and Castle, 1986) and appears to regulate RNA synthesis (Elghonemy et al., 2005; Khromykh et al., 2003; Tilgner et al., 2005). Besides the DEN2 3'SL, other sequences within the DEN2 3'UTR modulate viral translation and infectivity (Edgil et al., 2003), implicating the DEN 3'UTR in regulating viral translation in addition to RNA synthesis.

Recently, antisense peptide-conjugated phosphorodiamidate morpholino oligomers (P-PMOs) were utilized to inhibit DEN and WNV replication, presumably by sterically interfering with critical RNA–RNA or RNA–protein interactions involved in either viral translation or RNA synthesis (Deas et al., 2005; Kinney et al., 2005). PMOs are DNA-like oligomers, usually containing 18–25 bases, with six-member morpholine rings and phosphorodiamidate intersubunit linkages (Fig. 1A). They contain the same purine and pyrimidine bases as DNA for Watson-Crick base pairing with complementary target sequence. The water-soluble structure allows the PMO to base pair with RNA in a stable and sequence-specific manner without inducing RNase H degradation. An arginine-rich peptide conjugated to the 5' end of the P-PMOs allows for efficient uptake in cell culture (Moulton et al., 2004). A P-PMO complementary to the first 20 bases of the WNV RNA genome, named “5'END”, interfered with viral replication likely via inhibition of the translation process, and a P-PMO complementary to the WNV 3' cyclization region, named “3'CSI”, presumably acted by interfering with RNA synthesis (Deas et al., 2005). However, the mechanisms by which the analogous DEN-targeted P-PMOs, named “5'SL” and “3'CS”, inhibit DEN replication have not been determined (Kinney et al., 2005).

In this study, the DEN 5'SL and 3'CS P-PMOs were compared to a set of newly designed P-PMOs that were

complementary to conserved domains in the DEN 3'UTR to evaluate their relative effectiveness in inhibiting DEN replication. The mechanism by which the DEN-specific P-PMOs inhibited viral replication was explored using a DEN2 reporter mRNA and a newly constructed DEN2 reporter replicon. A novel P-PMO, complementary to the top of the DEN 3'SL, decreased viral replication apparently by inhibiting both viral translation and RNA synthesis. The DEN 5'SL P-PMO interfered only with viral translation, similar to the WNV 5'SL P-PMO (Deas et al., 2005). The DEN 3'CS P-PMO reduced viral RNA synthesis and did not inhibit viral translation, as did the WNV 3'CSI P-PMO (Deas et al., 2005). These DEN-specific P-PMOs are not only useful for dissecting critical domains involved in DEN translation and RNA synthesis but are also potentially useful as therapeutics for human infections.

## Results

### *Design of P-PMOs complementary to conserved domains of the DEN2 3'UTR*

In this study, P-PMOs were designed to be complementary to several conserved domains within the DEN2 3'UTR for the purpose of providing insight into how these domains regulate DEN replication. Two P-PMOs, “3'PKIIA” and “3'PKIIB”, were designed to potentially disrupt the RNA–RNA interaction of the two DEN2 pseudoknot structures (Fig. 1C; Table 1), which appear to play a role in regulating translation of DEN2 reporter mRNA (Holden and Harris, unpublished data). Another P-PMO, “3'CS2/RCS2”, was designed to base pair with either CS2 or RCS2 as the sequence of the P-PMO is complementary to both sequence elements. The DEN P-PMO, “3'SLT”, was designed to target the top of the 3'SL, including the highly conserved pentanucleotide loop (Wengler and Castle, 1986).

Two P-PMOs were used as positive controls in these studies, as they were recently shown to decrease DEN replication (Kinney et al., 2005). These two P-PMOs, “5'SL” and “3'CS”, are complementary to the first 20 nucleotides (nt) of the 5'UTR and to the 3'CS region, respectively. The first 69 nt of the flavivirus 5'UTR forms a stable conserved stem-loop structure (Brinton and Dispoto, 1988) that is necessary for viral replication (Cahour et al.,

Table 1  
Sequence of P-PMOs used in this study

P-PMO name <sup>a</sup>	Nucleotide sequence
DSer (negative control)	5'-AGT CTC GAC TTG CTA CCT CA-3'
5'SL	5'-GTC CAC GTA GAC TAA CAA CT-3'
5'AUG	5'-GGT TAT TCA TCA GAG ATC TG-3'
3'PKIIA	5'-TAC GCC ATG CGT ACA GCT TC-3'
3'PKIIB	5'-TTC CAG CGA GAC TAC AGC TTC-3'
3'CS2/RCS2	5'-GGT CTC CTC TAA CCT CTA GTC-3'
3'CS	5'-CCC AGC GTC AAT ATG CTG-3'
3'SLT	5'-CCA TTT TCT GGC GTT CTG TG-3'

<sup>a</sup> All P-PMOs used in this study are conjugated to R9F2 peptide.

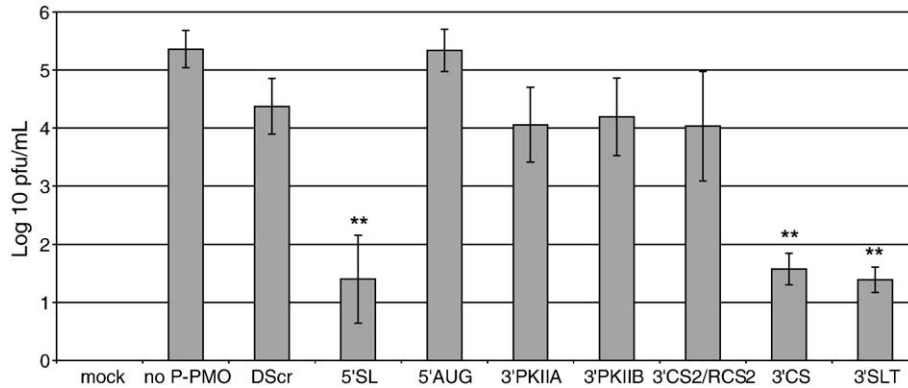


Fig. 2. Effects of P-PMOs on productive infection of BHK cells with DEN2 strain 16681. BHK cells were treated with 5  $\mu$ M P-PMO and infected with DEN2 strain 16681 at an MOI of 3. At 24 hpi, supernatants were harvested, and DEN titers were determined by viral plaque assay. Error bars indicate the standard error of the mean (SEM) of at least three independent experiments. DEN P-PMOs that significantly decreased viral titers compared to the DScr P-PMO ( $P < 0.05$ ) are indicated by \*\*.

1995), as is the 3'CS (Khromykh et al., 2001). Two P-PMOs were included as negative controls. The “DScr” P-PMO consisted of randomly generated sequence of 50% G/C content and lacks any significant complementarity to DEN sequence. This P-PMO acts as a control for non-specific cellular and viral effects of the P-PMO oligomer chemistry. The other negative control, the “5'AUG” P-PMO, is complementary to the region surrounding the start codon at the 5' end of the RNA genome and was previously shown to have minimal effect on DEN replication in Vero cells (Kinney et al., 2005).

#### The DEN 3'SLT P-PMO inhibits DEN2 viral replication in BHK cells

This panel of P-PMOs consists of the 5'SL, 5'AUG, 3'PKIIA, 3'PKIIB, 3'CS2/RCS2, 3'CS, 3'SLT, and DScr P-PMOs, and they were tested for their ability to inhibit a single cycle of DEN2 replication in BHK cells. Cells were pre-treated with 5  $\mu$ M of

P-PMO for 3 h before infection with DEN2 strain 16681 in the absence of P-PMO. Pre-treatment of cells with P-PMO was previously shown to be necessary for maximum antiviral activity (Kinney et al., 2005). After 2 h of DEN2 adsorption, virus was removed, and P-PMO-containing medium was added to the cells. At 24 h post-infection (hpi), supernatant was removed, and the amount of infectious virus was determined by viral plaque assay (Fig. 2). As previously demonstrated in Vero cells (Kinney et al., 2005), both the DEN 5'SL and 3'CS P-PMOs inhibited DEN2 replication by nearly 1000-fold in BHK cells (Fig. 2). The 3'SLT P-PMO also inhibited DEN2 replication by nearly 1000-fold. The other P-PMOs (3'PKIIA, 3'PKIIB, 3'CS2/RCS2) and the negative control P-PMOs (DScr and 5'AUG) lacked significant inhibitory activity against DEN2 replication in BHK cells (<10-fold). Similar results were obtained when the supernatant of infected P-PMO-treated cells was removed at 48 hpi (data not shown). Thus, these experiments identified a novel P-PMO targeting the top of the DEN 3'SL that robustly inhibits DEN2 replication.

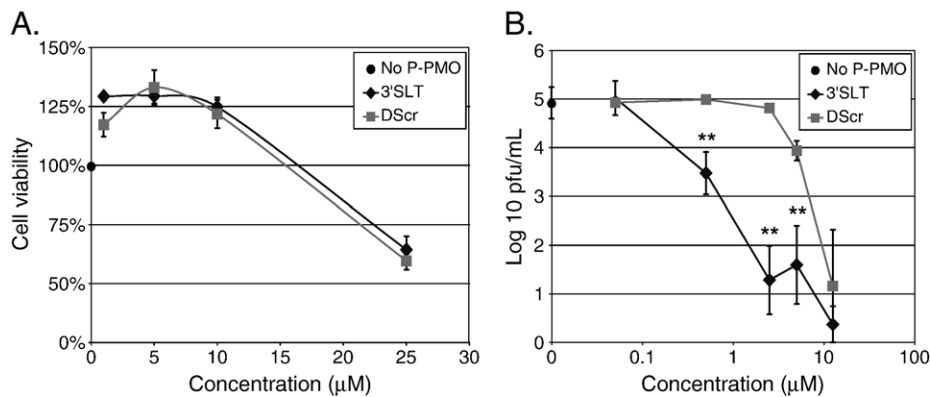


Fig. 3. Specific dose–response inhibition of DEN2 replication by the DEN 3'SLT P-PMO. (A) Cell viability in the presence of P-PMOs. BHK cells were incubated with different concentrations (1–25  $\mu$ M) of the DEN 3'SLT P-PMO and the DScr P-PMO for 24 h. Cell viability was determined using an MTS assay and compared to the absence of P-PMO (set at 100%). Error bars indicate the SEM of at least three independent experiments. No significant difference in cell viability was observed between the 3'SLT P-PMO and the DScr P-PMO at all concentrations. Both P-PMOs had a significant ( $P < 0.05$ ) negative impact on cell viability when present at 25  $\mu$ M. (B) Inhibition of DEN2 replication in a dose-dependent manner. BHK cells were infected with DEN2 strain 16681 at an MOI of 3 and treated with either the DEN 3'SLT P-PMO or the DScr P-PMO at concentrations between 0.05  $\mu$ M and 12.5  $\mu$ M. At 24 hpi, supernatants were harvested, and DEN titers were determined by viral plaque assay. Error bars indicate the SEM of at least three independent experiments. Concentrations of the DEN 3'SLT P-PMO that resulted in significantly different titers compared to treatment with the DScr P-PMO ( $P < 0.05$ ) are indicated by \*\*.

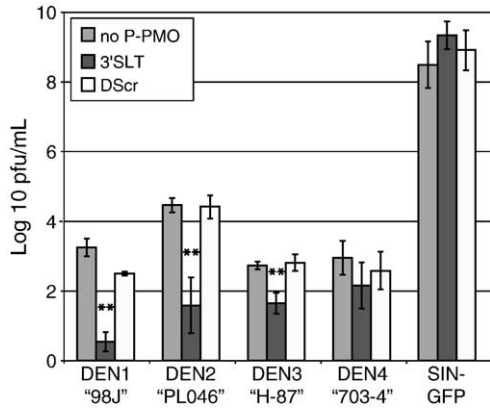


Fig. 4. Specificity of inhibition of viral infection by the DEN 3'SLT P-PMO. BHK cells were treated with either the DEN 3'SLT P-PMO or the DScr P-PMO (2.5 μM) and infected with DEN1 strain 98J (MOI of 0.5), DEN2 strain PL046 (MOI of 3), DEN3 strain H-87 (MOI of 0.5), DEN4 strain 703-4 (MOI of 0.5), or SIN-GFP (MOI of 3). Lower MOIs were used for DEN1, DEN3, and DEN4 due to the lower titers obtained for these serotypes. At 24 hpi, supernatants were harvested, and DEN or SIN titers were determined by viral plaque assay. Error bars indicate the SEM of at least three independent experiments. Viral titers that were significantly different ( $P < 0.05$ ) after treatment with the DEN 3'SLT P-PMO as compared to the DScr P-PMO are indicated by \*\*.

*The DEN 3'SLT P-PMO specifically inhibits viral replication at low concentrations*

To determine which concentrations of the DEN 3'SLT P-PMO are both non-cytotoxic and effective, a range of concentrations were tested in a cell proliferation assay and against DEN2 replication. First, cells were treated with a range of P-PMO concentrations for 24 h before cell proliferation was tested using an MTS tetrazolium salt assay that measures NADH and NADPH produced by living cells (Fig. 3A). Cell proliferation was non-specifically inhibited at 25 μM of the 3'SLT or DScr P-PMO. Ten micromolars or less of either P-PMO had no effect on cellular growth. Next, the inhibition of DEN2 replication due to various concentrations of the 3'SLT and DScr P-PMOs was evaluated (Fig. 3B). Cells were treated with either of the two P-PMOs both before and after DEN2 infection. Virus production was determined by performing viral plaque assays on the supernatant at 24 hpi. Significant inhibition of DEN2 replication was detected when the 3'SLT P-PMO concentrations were between 0.5 μM and 5 μM. The DScr P-PMO generated non-specific inhibition of DEN2 replication at a

concentration of 12.5 μM. The greatest difference between the effect of the 3'SLT P-PMO and the DScr P-PMO on DEN2 replication occurred at 2.5 μM. Therefore, between 0.5 μM and 5 μM of the 3'SLT P-PMO was sufficient to specifically inhibit DEN2 replication without impairing normal cellular processes.

*BHK cells take up P-PMOs and are infected with DEN2 efficiently*

To determine the efficiency of P-PMO uptake by BHK cells at a concentration (2.5 μM) at which the 3'SLT P-PMO is both non-cytotoxic (Fig. 3A) and effective (Fig. 3B), the DScr P-PMO was 3' end-labeled with fluorescein and added to BHK cells before and after DEN2 infection (Supplementary Figure). The ability of the cells to become productively infected was also monitored by immunofluorescence with antibodies against the DEN NS3 protein. At 24 hpi, over 90% of the cells contained the fluorescein-labeled P-PMO. In addition, over 90% of the cells were productively infected with DEN2. Thus, nearly all of the cells both contained the fluoresceinated P-PMO and were productively infected with DEN2.

*The DEN 3'SLT P-PMO specifically inhibits DEN types 1, 2, and 3*

The ability of the 3'SLT P-PMO to inhibit other DEN strains and serotypes as well as an alphavirus was evaluated in BHK cells to further assess the specificity of the 3'SLT P-PMO. Cells were treated with the 3'SLT or DScr P-PMO and infected with different DEN serotypes or with Sindbis virus (SIN), and viral replication was monitored by viral plaque assay (Fig. 4). The 3'SLT P-PMO inhibited viral replication of another DEN2 strain (PL046) over 600-fold, similar to the effect on DEN2 strain 16681. Similarly, it reduced viral replication of DEN1 strain 98J and DEN3 strain H-87 by ~80-fold and ~15-fold, respectively. The sequence of the DEN 3'SLT P-PMO was perfectly complementary to the sequence of DEN types 1, 2, and 3 strains used in this study (Table 2). DEN4 strain 703-4 was not specifically inhibited by the 3'SLT P-PMO, probably due to five mismatched base pairs in the target region in this DEN4 strain (Table 2). The 3'SLT P-PMO had no effect on the replication of SIN, indicating that this P-PMO is specific for DEN and does not

Table 2  
Alignment of DEN1–4 strains used in this study with the complement of the DEN 3'SLT P-PMO

	Strain <sup>a</sup>	Accession #	Sequence (5'–3')
DEN1	98J	Unpublished <sup>b</sup>	CAC AGA ACG CCA GAA AAU GG <sup>c</sup>
DEN2	16681	U87411	-----
DEN2	PL046	Unpublished <sup>b</sup>	-----
DEN3	H-87	M93130	-----
DEN4	703-4	AF310153	----- G----G C-- G-- C-

<sup>a</sup> DEN strains used in this study.

<sup>b</sup> S. Shresta, D. Prigozhin, and E. Harris, unpublished data.

<sup>c</sup> Sequence is an exact complement to the sequence of the DEN 3'SLT P-PMO. Dashes indicate same nt as the first sequence reported.

display antiviral activity against a non-flavivirus. Thus, the 3'SLT P-PMO inhibited viral replication only if the complementary sequence with few mismatches (<5) was present in the viral genome.

#### DEN-specific P-PMOs inhibit viral translation and/or RNA synthesis

P-PMOs are hypothesized to inhibit either viral translation or RNA synthesis by steric interference with critical RNA–protein or RNA–RNA interactions involved in these processes. The three DEN-specific P-PMOs (5'SL, 3'CS, and 3'SLT) were assessed for their effect on viral translation and RNA synthesis. For positive-strand RNA viruses, these two viral processes are dependent on each other; without translation of the viral genome, RNA synthesis cannot occur, and without viral RNA synthesis, viral translation can only proceed linearly until the RNA is degraded. To determine whether each DEN-specific P-PMO inhibited viral replication through one or both of these processes, viral protein and RNA levels were monitored in DEN2-infected cells in the presence or absence of the DEN-specific P-PMOs or the DScr P-PMO.

Viral RNA was quantified by real-time RT-PCR in which a region of the NS1 gene was amplified from both the positive- and negative-sense DEN2 RNA strands present at 2, 6, 16, and 24 hpi (Fig. 5A). The DEN-specific P-PMOs and the DScr P-PMO had no effect on the amount of viral RNA (Fig. 5A) or on the amount of cellular  $\beta$ -actin mRNA (data not shown) present at 2 hpi as compared to when no P-PMO was present, indicating that viral entry was not impacted by the P-PMOs. The DScr P-PMO non-specifically inhibited viral RNA production at 16 hpi but resulted in only a four-fold reduction in viral RNA levels at 24 hpi. Both the 5'SL P-PMO and the 3'CS P-PMO inhibited viral replication, resulting in a 20-fold and 60-fold reduction in viral RNA by 24 hpi, respectively, as compared to the “no P-PMO” control. The 3'SLT P-PMO was the most effective P-PMO tested, reducing viral RNA levels over 450-fold compared to when no P-PMO was present during infection. No differences in the amount of cellular SDHA mRNA existed at 24 hpi between the different conditions (data not shown), suggesting that the DEN-specific P-PMOs were specifically impacting viral RNA synthesis. The reduction of viral RNA was similar in magnitude to the reduction in virus progeny released at 24 hpi (Figs. 2, 5A; data not shown), indicating that the differences in viral titers were not due to the P-PMO blocking the release of viral progeny from infected cells.

To confirm the observed effect on viral RNA synthesis at 24 hpi, viral protein production at 24 hpi was detected by Western blot analysis using anti-NS1 antibodies when no P-PMO, a DEN-specific P-PMO, or the DScr P-PMO was present during a DEN2 infection (Fig. 5B). The DScr P-PMO caused a slight non-specific reduction in the amount of viral protein at 24 hpi. The 5'SL, 3'CS, and 3'SLT P-PMOs each inhibited viral replication such that NS1 was below the limit of detection ( $\geq 25$ -fold difference). The differences in the amount of NS1 were also not due to differences in cell number or gel loading as similar amounts of actin were detected in each lane on the

same blot. Therefore, the DEN-specific P-PMOs most likely inhibit DEN replication by interfering with viral translation and/or viral RNA synthesis, and not by affecting virus entry into the cells or virus maturation and egress.

#### DEN-specific P-PMOs regulate translation of a DEN2 reporter mRNA

The data presented thus far indicate that the DEN-specific P-PMOs may affect viral translation and/or RNA synthesis, thereby reducing overall viral replication. To test whether the P-PMOs affect viral translation independently from viral RNA

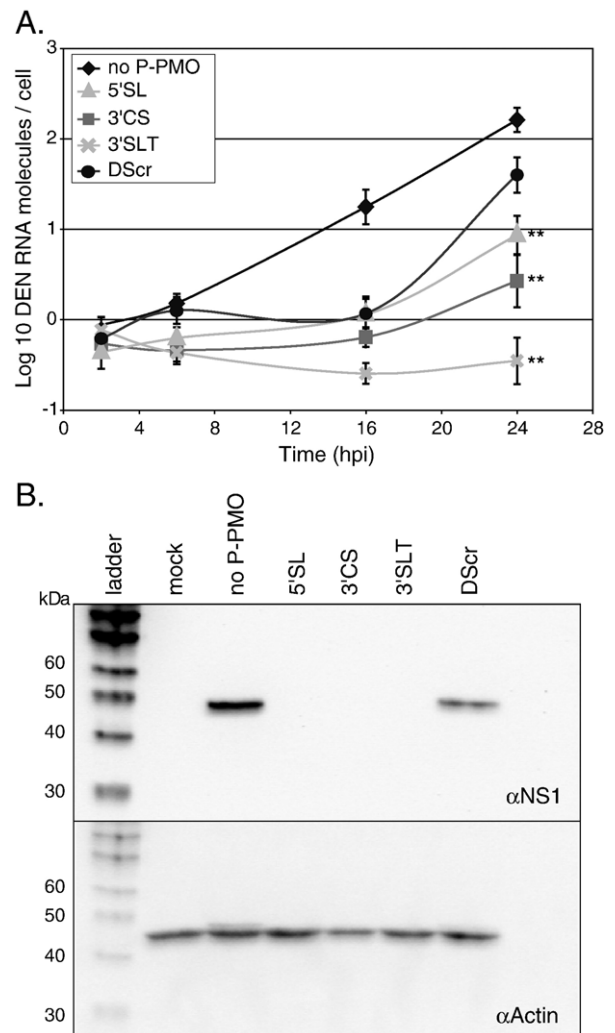


Fig. 5. DEN-specific P-PMOs inhibit viral translation and/or RNA synthesis. BHK cells were infected with DEN2 strain 16681 and treated with the DEN-specific P-PMOs (5'SL, 3'CS, and 3'SLT) or the DScr P-PMO. (A) Effects of the DEN-specific P-PMOs on viral RNA accumulation. Cells were harvested at 2, 6, 16, and 24 hpi, and total RNA was isolated. DEN RNA strands were quantified by real-time RT-PCR using NS1-specific primers. Error bars indicate the SEM of at least three independent experiments. P-PMOs that resulted in significantly different numbers of DEN RNA molecules at 24 hpi as compared to the DScr P-PMO ( $P < 0.05$ ) are indicated by \*\*. (B) Effects of the DEN-specific P-PMOs on viral protein accumulation. Cells harvested at 24 hpi were analyzed by Western blot hybridization using anti-NS1 antibodies and were re-probed using anti-actin antibodies. The limit of detection is  $\geq 25$ -fold. A representative Western blot of three separate experiments is shown.

synthesis, a previously described DEN2 reporter mRNA (“5’DEN-LUC-3’DEN”; Fig. 6A) (Holden and Harris, 2004) was used, which consists of the DEN2 5’UTR plus the first 72 nt of the capsid (C) gene and the DEN2 3’UTR flanking the firefly luciferase reporter gene. The beginning of C was included because it contains the 5’CS, which is complementary to the 3’CS present in the DEN 3’UTR. BHK cells were treated with a DEN-specific P-PMO, no P-PMO, or the DScr P-PMO both before and after RNA transfection of the DEN2 reporter mRNA. RNA transfection efficiency and the stability of the DEN2 reporter mRNA, as measured using a quantitative real-time RT-PCR assay, were not affected by treatments with the different P-PMOs (data not shown). Luciferase activity was monitored for up to 8 h post-transfection (Fig. 6B, Table 3; the data in Fig. 6B are representative of three independent experiments whose average values are presented in Table 3). The DScr P-PMO had a slight enhancing effect on translation early after RNA transfection; however, by 8 h post-transfection, this P-PMO had no effect on the translation of the DEN2 reporter mRNA compared to when no P-PMO was present. The 5’SL P-PMO inhibited translation of the DEN2 reporter mRNA, reducing translation efficiency by 95% at 8 h post-transfection compared to either the absence of P-PMO or to the

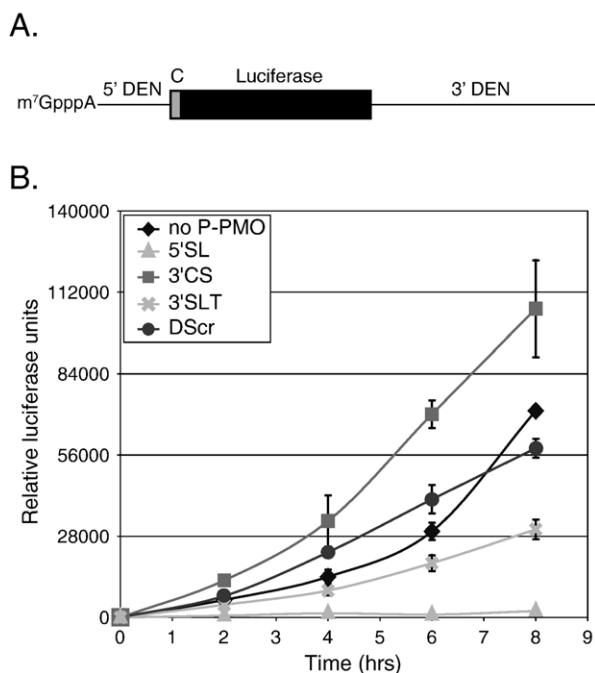


Fig. 6. Effects of the DEN-specific P-PMOs on translation of the DEN2 reporter mRNA. (A) Schematic diagram of the DEN2 reporter mRNA. Indicated are the DEN2 5’UTR (black line), first 72 nt of C (gray box) fused to the firefly luciferase gene (black box), and the DEN2 3’UTR (black line). (B) DEN-specific P-PMOs affect translation of the DEN2 reporter mRNA. BHK cells were treated with the DEN-specific P-PMOs (5’SL, 3’CS, and 3’SLT) or the DScr P-PMO then transfected with the DEN2 reporter mRNA. P-PMOs were added back after RNA transfection. Luciferase activity was monitored at 2, 4, 6, and 8 h after transfection. Data shown are representative of three independent experiments. Error bars indicate the standard deviation (SD) of duplicate samples. The amount of transfected DEN2 reporter mRNA and the stabilities of the DEN2 reporter mRNAs were not impacted by the presence of the P-PMOs, as determined by quantitative real-time RT-PCR (data not shown).

Table 3

Average translation efficiency of the DEN2 reporter mRNA in the presence of the DEN-specific P-PMOs

	Translation Efficiency (8 h) <sup>a</sup>
No P-PMO	100
DScr P-PMO	100 ± 10
5’SL P-PMO	5 ± 1 <sup>b</sup>
3’CS P-PMO	265 ± 60
3’SLT P-PMO	47 ± 3 <sup>b</sup>

<sup>a</sup> To calculate translation efficiency, the luciferase activity was normalized to the amount of relative luciferase units produced in the absence of P-PMO 8 h after transfection in three independent experiments. SEM is shown.

<sup>b</sup> Significantly different from the luciferase activity in the presence of the DScr P-PMO ( $P < 0.05$ ).

presence of the DScr P-PMO. The 3’CS P-PMO appeared to enhance translation of the DEN2 reporter mRNA over 2.5-fold compared to either the DScr P-PMO or no P-PMO. In contrast, the 3’SLT P-PMO reduced the translation efficiency of the DEN2 reporter mRNA by 50% compared to when no P-PMO or the DScr P-PMO was present. These results suggest that both the regions targeted by the 5’SL P-PMO and the 3’SLT P-PMO are required for efficient translation of the DEN2 reporter mRNA.

#### Construction and characterization of a novel DEN2 reporter replicon

To distinguish between effects of DEN-specific P-PMOs on viral translation and/or RNA synthesis, a DEN2 reporter replicon “DEN2rep-FH” (Fig. 7A) was constructed. The DEN2 infectious clone (Kinney et al., 1997) (pD2/IC, gift of R. Kinney, Center for Disease Control and Prevention, Fort Collins, CO) was used as the backbone to construct the DEN2 reporter replicon, in which the firefly luciferase gene replaced most of the structural genes (see Materials and methods). The DEN2 reporter replicon should translate and replicate in a manner similar to wild-type DEN RNA. The DEN2 reporter replicon is not infectious because it lacks the coding sequences for structural proteins, which are necessary for assembly. The DEN2 reporter replicon was transcribed in vitro and transfected into BHK cells. Luciferase activity was monitored for 96 h post-transfection (Fig. 7B). Luciferase activity peaked within the first 8 h, and, by 24 h after transfection, luciferase activity was significantly reduced. Between 48 and 96 h post-transfection, luciferase activity increased significantly and to a greater extent than during the first 12 h. Thus, two peaks of luciferase activity were generated by the DEN2 reporter replicon: an early peak between 4 and 8 h post-transfection and a later peak between 48 and 96 h post-transfection.

Mycophenolic acid (MPA), a potent inhibitor of DEN RNA synthesis (Diamond et al., 2002), was added after RNA transfection of the DEN2 reporter replicon to determine whether the first or second peak of luciferase activity corresponds to RNA synthesis of the DEN2 reporter replicon (Fig. 7B). MPA significantly inhibited only the appearance of the later peak (48–96 h post-transfection) and not the first peak

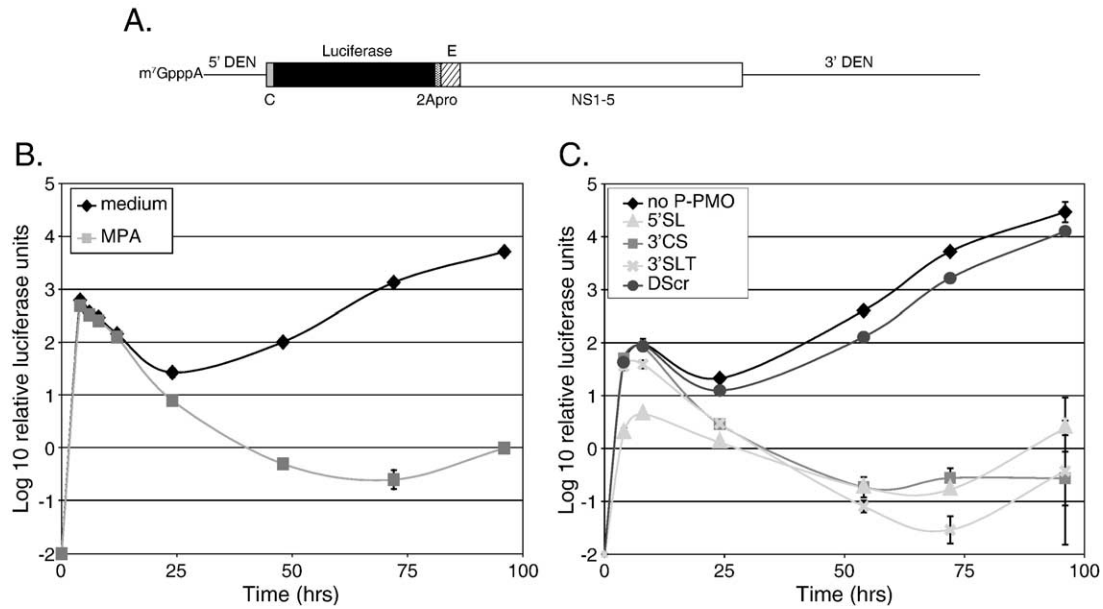


Fig. 7. Effects of the DEN-specific P-PMOs on translation and RNA synthesis of the DEN2 reporter replicon. (A) Schematic diagram of the DEN2 reporter replicon. Indicated are the DEN2 5'UTR (black line), first 72 nt of C (gray box) fused to the firefly luciferase gene (black box), the FMDV 2Apro (dotted box), the last 90 nt of E (striped box), the entire NS region (white box), and the DEN2 3'UTR (black line). (B) The DEN2 reporter replicon distinguishes between viral translation and RNA synthesis. Cells were transfected with the DEN2 reporter mRNA then treated with medium or MPA (3  $\mu$ M). Luciferase activity was monitored at 4, 8, 24, 54, 72, and 96 h after transfection. Data shown are representative of three independent experiments. Error bars indicate the SD of duplicate samples. The amount of transfected RNA was not impacted by the presence of the P-PMOs, as determined by quantitative real-time RT-PCR (data not shown). (C) DEN-specific P-PMOs inhibit translation and/or RNA synthesis of the DEN2 reporter replicon. Cells were treated with the DEN-specific P-PMOs (5'SL, 3'CS, and 3'SLT) or the DScr P-PMO and transfected with the DEN2 reporter replicon. Luciferase activity was monitored at 4, 6, 8, 12, 24, 48, 72, and 96 h after transfection. Data shown are representative of three independent experiments. Error bars indicate the SD of duplicate samples. The amount of transfected RNA was not impacted by the presence of P-PMO, as determined by quantitative real-time RT-PCR (data not shown).

of luciferase activity (4–8 h). Additionally, MPA reduced the amount of DEN2 reporter replicon RNA present at 24 h post-transfection, as determined by quantitative real-time RT-PCR (data not shown). By inhibiting RNA synthesis, MPA also interfered with the translation of the DEN2 replicon at later times post-transfection. This indicates that the first peak of luciferase activity (4–8 h) correlates with translation of the DEN2 reporter replicon, whereas the later peak of luciferase activity (48–96 h) is dependent upon RNA synthesis.

#### *DEN-specific P-PMOs inhibit translation and RNA synthesis of a DEN2 reporter replicon*

The DEN2 reporter replicon was then used to distinguish between the effect of the DEN-specific P-PMOs on viral translation and RNA synthesis. Cells were treated with the different P-PMOs before transfection of the DEN2 reporter replicon and again after RNA transfection (Fig. 7C, Table 4; the data in Fig. 7C are representative of three independent experiments whose average values are presented in Table 4). The different P-PMOs had no effect on RNA transfection efficiency as determined by real-time RT-PCR (data not shown). Luciferase activity was monitored for 96 h. The DScr P-PMO had a slightly positive effect (24% increase) on the translation of the DEN2 reporter replicon at 8 h and a negative impact (40% decrease) on the RNA synthesis peak of the replicon RNA at 96 h, as compared to the “no P-PMO” control. The 5'SL P-PMO, which inhibited translation of the

DEN reporter mRNA (Fig. 6B; Table 3), also inhibited translation of the DEN2 reporter replicon by 95% (Fig. 7C; Table 4). As a result, the 5'SL P-PMO inhibited the appearance of the RNA synthesis peak by over 10,000-fold. The 3'CS P-PMO slightly enhanced translation of the replicon (17%) but strongly inhibited the appearance of the RNA synthesis peak of the DEN2 reporter replicon ( $\geq 10,000$ -fold), indicating that the 3'CS P-PMO inhibited only viral RNA synthesis. The 3'SLT P-PMO reduced translation of the DEN2 reporter replicon by 40% and inhibited the second peak of luciferase activity that is primarily dependent on RNA synthesis by over 10,000-fold.

Table 4

Average translation and replication efficiency of the DEN2 reporter replicon in the presence of the DEN-specific P-PMOs

	Translation efficiency (8 h) <sup>a</sup>	Replication efficiency (96 h) <sup>a</sup>
No P-PMO	100	100 <sup>b</sup>
DScr P-PMO	136 $\pm$ 23	38 $\pm$ 5
5'SL P-PMO	5 $\pm$ 1 <sup>b</sup>	0.006 $\pm$ 0.001 <sup>b</sup>
3'CS P-PMO	125 $\pm$ 14	0.003 $\pm$ 0.001 <sup>b</sup>
3'SLT P-PMO	59 $\pm$ 9 <sup>b</sup>	0.006 $\pm$ 0.001 <sup>b</sup>

<sup>a</sup> To calculate translation efficiency, the luciferase activity was normalized to the amount of relative luciferase units produced in the absence of P-PMO 8 h and 96 h after transfection in three independent experiments. Replication efficiency is dependent on both translation and RNA synthesis efficiency. SEM is shown.

<sup>b</sup> Significantly different from luciferase activity in the presence of the DScr P-PMO ( $P < 0.05$ ).

The 3'SLT P-PMO did not inhibit translation of the DEN2 reporter replicon to as great a degree as the 5'SL P-PMO (2-fold vs. 20-fold); however, the 3'SLT P-PMO reduced the ability of the DEN2 replicon RNA to synthesize more replicon RNA as effectively as the 5'SL P-PMO did. The significant reduction in the replication of the DEN2 reporter replicon by 3'SLT P-PMO indicates that it directly inhibits RNA synthesis in addition to its effects on translation.

## Discussion

Antisense molecules that disrupt RNA–RNA or RNA–protein interactions that are critical for viral replication are potentially useful both as therapeutic treatments and as tools to explore *cis*-acting elements involved in viral translation and/or RNA synthesis. In this study, an antisense P-PMO complementary to the top of the terminal, conserved DEN2 3'SL, which includes a highly conserved flavivirus pentanucleotide loop, inhibited viral replication in BHK cells. At a low concentration, the DEN 3'SLT P-PMO was efficiently taken up by cells and proved to be non-cytotoxic and effective. The DEN 3'SLT P-PMO reduced viral replication of DEN types 1, 2, and 3, but not DEN4, likely due to mismatched base pairs between the P-PMO and target sequence. It appears that the DEN 3'SLT P-PMO inhibited DEN replication by interfering with both viral translation and RNA synthesis.

Several other P-PMOs were designed to bind to conserved domains in the DEN2 3'UTR. Two pseudoknot (PK) structures, PKIIA and PKIIB, were targeted because they may be involved in regulating viral translation (K.L. Holden and E. Harris, unpublished data). PKIIA and PKIIB contain loops that presumably bind to upstream sequences, forming pseudoknot structures (Olsthoorn and Bol, 2001). The DEN PKIIB and PKIIA also contain nearly identical conserved sequences of 23 nt, called CS2 and RCS2, respectively, which play a role in regulating RNA synthesis (Lo et al., 2003). P-PMOs targeting either the loops or the CS2/RCS2 sequences of both PKs were not effective in reducing viral titers as compared to the negative control DScr P-PMO. The inability of these P-PMOs to block viral replication may be due to (1) inefficient binding to the target sequence, (2) compensation of the disrupted element by a redundant structure, or (3) lack of a regulatory role in DEN2 replication by the PK domains *in vitro*. To differentiate among these hypotheses, these P-PMOs could be redesigned, tested in combination, and/or evaluated in a small animal model.

The effect of both the 5'SL- and the 3'CS-targeted P-PMOs on DEN and WNV replication was previously reported (Deas et al., 2005; Kinney et al., 2005). These two P-PMOs were effective in inhibiting viral replication in monkey (Vero) cells (Kinney et al., 2005) and in hamster (BHK) cells (Deas et al., 2005). The 5'SL, 3'CS, and 3'SLT P-PMOs tested in our study were found to have a negative impact on DEN replication in BHK cells, complementing the previous studies. The mechanism of inhibition of DEN replication was not previously investigated for the 5'SL and 3'CS P-PMOs (Kinney et al., 2005). A 5'SL-targeted P-PMO was found to inhibit WNV

translation, while the 3'CSI P-PMO inhibited WNV RNA synthesis without affecting viral translation (Deas et al., 2005), and our studies of DEN support these WNV findings.

A P-PMO complementary to the top loop of the WNV 3'SL (“3'Loop” P-PMO) was shown previously to moderately inhibit viral replication by an uncharacterized mechanism (Deas et al., 2005), whereas the DEN 3'SLT P-PMO inhibited DEN replication as effectively as the 5'SL or 3'CS P-PMOs in the present study. We found that the DEN 3'SLT P-PMO reduced the efficiency of both viral translation and RNA synthesis. The difference between the results for the WNV 3'Loop P-PMO and for the DEN 3'SLT P-PMO may be due to slight differences in the target sequence of these P-PMOs or to differences in how dependent these viruses are on the 3'SL for viral translation. Deletion of the entire WNV 3'UTR was reported to have no effect on the translation of a WNV replicon (Tilgner et al., 2005), and the presence of the WNV 3'SL was found to have a negative impact on translation of a WNV reporter mRNA (Li and Brinton, 2001). In contrast, the deletion of the DEN2 3'SL was shown to decrease translation efficiency of a DEN2 reporter mRNA (Holden and Harris, 2004), and, in data presented in this study, the DEN 3'SLT P-PMO inhibited DEN translation of both a DEN2 reporter mRNA and a DEN2 reporter replicon. In addition, a DEN2 replicon containing mutations in the 3'SLT P-PMO binding region also demonstrated a significant decrease in translation efficiency (K. Clyde, K. Holden, and E. Harris, unpublished data), further implicating the 3'SL region in the regulation of DEN translation. It is possible that the 3'UTR of these two flaviviruses control viral translation by somewhat different mechanisms and/or that the DEN 3'UTR is more critical for regulation of viral translation than the WNV 3'UTR.

A positive-sense RNA virus genome must negotiate between being translated by ribosomes starting from the 5' end and being replicated to generate negative-sense RNA by the viral replicase initiating at the 3' end. Otherwise, a collision of translating ribosomes and the viral replicase would result in premature termination of either or both processes. For example, poliovirus coordinates viral translation and RNA synthesis by overlapping regulatory domains within its 5'UTR (Gamarnik and Andino, 1998). In the case of DEN, the appearance of two distinct peaks in reporter gene expression for the DEN2 reporter replicon suggests that, up to 8 h post-transfection, translation occurs, as expected, but at approximately 24 h post-transfection, translation is suppressed while viral RNA synthesis is favored. After a sufficient amount of viral RNA is present, viral protein synthesis resumes. Switching between the opposing processes of viral translation and RNA synthesis likely involves the viral UTRs.

It is possible that as a DEN infection progresses within a cell, the DEN 3'CS and 3'SL, which are in close proximity to each other in the DEN 3'UTR, may facilitate a switch between viral translation and RNA synthesis and may be regulated by the relative amounts of viral proteins and viral negative- and positive-strand RNAs. Initially, when no viral proteins are present, the 3'SL may act to enhance viral translation at initiation (Holden and Harris, 2004), possibly via an interaction with the

cellular translation machinery. As the amount of viral proteins accumulates to form the viral replicase, the DEN 3'SL may switch from enhancing viral translation to promoting viral RNA synthesis via an RNA–RNA or RNA–protein interaction. A rearrangement of the viral RNA structure at the 3' end may allow the 3'CS to base pair with the 5'CS to promote viral RNA synthesis. Concurrently, the 3'UAR, which is located in the lower stem of the 3'SL, may base pair with the 5'UAR to disrupt the 3'SL structure (Alvarez et al., 2005). After a sufficient amount of viral RNA synthesis, the base pairing interactions between the 5' and 3' ends of the DEN genome may no longer be favored, and the DEN 3'SL may switch back to promoting viral translation. Therefore, the DEN 3'CS and 3'SL, possibly in conjunction with other elements in the DEN 5'- and 3'-UTRs, may facilitate either DEN translation or RNA synthesis, depending on RNA–RNA and/or RNA–protein interactions.

An antisense phosphorothioate oligomer that blocks the translation of CMV immediate early 2 (IE2) mRNA has been approved by the Federal Drug Administration as an antiviral therapeutic to treat CMV retinitis (Marwick, 1998), indicating that antisense molecules are feasible as therapeutics for viral infections. Antisense molecules of various chemistries have been demonstrated to inhibit other viral infections (Ma et al., 2000), including DEN in monkey LLCMK/2 cells (Raviprakash et al., 1995). In the earlier DEN study, two phosphorothioate antisense oligos complementary to a sequence near the 5' start codon and to a sequence near the top of the 3'SL, but excluding the conserved pentanucleotide loop, moderately inhibited DEN replication (Raviprakash et al., 1995).

Dengue is an important public health problem worldwide. Because no vaccines or antiviral therapies are currently available to prevent or cure DEN infections, research that utilizes antisense or other methodologies to prevent infection or reduce viremia is necessary. The information acquired from previous research (Deas et al., 2005; Kinney et al., 2005; Raviprakash et al., 1995) and this study on antisense molecules may lead to the development of antiviral compounds that are efficacious, inexpensive, and well-tolerated to treat DEN infections and other flavivirus infections.

## Materials and methods

### Cell lines and viruses

BHK-21 (baby hamster kidney) clone 15 cells were obtained from B. Childs (Center for Vector-borne Diseases, University of California, Davis, CA). BHK cells were grown at 37 °C and 5% CO<sub>2</sub> and were cultured in  $\alpha$ MEM supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA) and 10 mM HEPES. All tissue culture media and supplements were purchased from Invitrogen (Carlsbad, CA) except where noted. Cells were passaged every 3–4 days using HBSS with 3 mM EDTA and 10 mM HEPES to suspend the monolayer. C6/36 cell line was obtained from ATCC (Manassas, VA). C6/36 cells were maintained in L15 plus 10% FBS, 10 mM HEPES, and 100 U/ml penicillin and 100 mg/ml

streptomycin (Pen/Strep) at 28 °C without CO<sub>2</sub>. DEN was propagated in C6/36 cells (see below).

DEN1 strain 98J (a 1998 clinical isolate from Guyana), DEN2 strains 16681 (obtained from Center for Disease Control and Prevention (CDC), Fort Collins, CO) and PL046 (Taiwanese clinical isolate, gift of Dr. H.Y. Lei, National Cheng Kung University, Taiwan), DEN3 strain H-87 (CDC), and DEN4 strain 703-4 (CDC) were grown in C6/36 cells and titered using BHK cells as previously described (Diamond et al., 2000). DEN viral plaques were allowed to develop for 5–6 days prior to fixation with 10% formaldehyde and staining with 1% crystal violet in 30% ethanol, after which plaque forming units (pfu) per milliliter were calculated. A plasmid containing the Sindbis genome plus green fluorescent protein (GFP) (Frolova et al., 2002) was provided by Dr. Ilya Frolov (University of Texas Medical Branch, Galveston, TX). The plasmid was linearized with *Xho*I and transcribed in vitro using RiboMAX Large Scale RNA production System-SP6 (Promega, Madison, WI) in the presence of m<sup>7</sup>GpppG cap analog (New England BioLabs, Beverly, MA). The SIN-GFP RNA was electroporated into BHK cells, and virus was harvested at 24 h post-transfection, as previously described (Frolova et al., 2002). SIN-GFP virus was titered by viral plaque assay as described above, except that they were fixed 2 days post-infection.

### Computer-predicted secondary structures

The secondary structures of the DEN2 strain 16681 5'UTR and 3'UTR were predicted using the *mfold* web server at [www.bioinfo.rpi.edu/applications/mfold/old/rna/](http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) under standard conditions (37 °C) (Zucker, 2003).

### P-PMO design and synthesis

An arginine-rich peptide NH<sub>2</sub>-RRRRRRRRRFFC-NH<sub>2</sub> (R9F2C) was covalently conjugated to the 5' end of each P-PMO (Fig. 1A). The synthesis, conjugation, purification, and analysis of R9F2C-PMO (abbreviated P-PMO in this report) compounds were similar to procedures described previously (Moulton et al., 2004; Summerton and Weller, 1997). Sequences in the DEN2 positive-strand genomic RNA located in regions within the DEN2 3'UTR found to be important in the DEN life-cycle (Elghonemy et al., 2005; Holden and Harris, 2004; Khromykh et al., 2001, 2003; Lo et al., 2003; Tilgner et al., 2005; You and Padmanabhan, 1999; Zeng et al., 1998; K.L. Holden and E. Harris, unpublished data) were chosen to design complementary P-PMO compounds of 18–21 bases in length. Sequences, name designations, and target locations of the P-PMOs are described in Table 1 and Figs. 1B and C. The DScr P-PMO, a randomly generated 20-mer P-PMO with 50% G/C content, was prepared to control for non-sequence-specific activity of the R9F2-PMO chemistry. To preclude any unintentional hybridization events, antisense and negative control P-PMO sequences were screened using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) against all primate mRNA and flaviviral sequences. Lyophilized P-PMOs were diluted to 2 mM using filter-sterilized distilled water and were stored at 4 °C.

### *Viral infections*

BHK cells were seeded in 12-well plates in  $\alpha$ MEM with 10% FBS and incubated until the cells were 50–80% confluent. Cells were washed twice with Virus Production-Serum Free Media (VP-SFM). Serum-free media is necessary for efficient uptake of R9F2-PMOs (Neuman et al., 2004). One milliliter of VP-SFM with the indicated concentration of P-PMO was added to duplicate wells. Cells were incubated for 2–3 h and washed twice with VP-SFM before viral stocks were added at the indicated MOI in 200  $\mu$ l VP-SFM. After 2-h incubation, cells were washed twice with VP-SFM, and 1 ml of VP-SFM with the indicated P-PMO was added back to each well. After a 4-h incubation, FBS was added at a concentration of 2% (except for the experiments represented in Fig. 2, where no serum was added back), and, 24 h after viral infection, the supernatant was removed and stored at  $-80^{\circ}\text{C}$  until viral plaque assays were performed.

### *Cell proliferation assay*

The linear range of BHK cell proliferation was determined by plating BHK cells in 100  $\mu$ l of  $\alpha$ MEM supplemented with 10% FBS at a density of  $5 \times 10^3$  to  $7 \times 10^5$  cells per well in a 96-well plate. Cells were incubated overnight before they were washed in VP-SFM and returned to the incubator for 1 h. CellTiter 96 Aqueous One Solution Reagent (Promega), which contains an MTS tetrazolium salt to measure NADPH and NADH produced from metabolically active cells, was added to each well as per the manufacturer's protocol. Cells were incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 2 h before the absorbance was read at 490 nm using an Elx808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT) and KCjunior software (Bio-Tek Instruments, Inc.). To assess the effect of P-PMOs on cell growth, BHK cells were plated at  $4 \times 10^4$  cells per well in a 96-well plate in 100  $\mu$ l  $\alpha$ MEM with 10% FBS and were incubated for 4 h. VP-SFM (100  $\mu$ l) with the indicated concentration of P-PMO was added to wells in quadruplicate. Cells were incubated for 24 h, at which point CellTiter 96 Aqueous One Solution Reagent (20  $\mu$ l) was added to each well and processed as described above. The average background (medium alone) was subtracted from each well. Cell viability was calculated by setting the absorbance reading from cells that were not treated with P-PMO to 100%.

### *Immunocytochemistry*

BHK cells were plated, treated with 3'-end fluorescein labeled DScr P-PMO, and infected with DEN2 strain 16681 as described above, except that coverslips were added to each well before plating the cells. At 24 hpi, cells were washed twice with PBS and fixed in 4% paraformaldehyde, pH 7.3 for 10 min (min) at room temperature (RT). Cells were washed twice with PBS before blocking with 5% normal goat serum (NGS) in HBSS supplemented with 10 mM HEPES and 0.1% saponin (H/HBSS/S) for 1 h at RT. Cells were incubated with primary antibody (monoclonal antibody anti-NS3 E1.E6 at a 1:1000

dilution) (P.R. Beatty, and E. Harris, unpublished data) in H/HBSS/S plus 5% NGS overnight at  $4^{\circ}\text{C}$  followed by three washes with H/HBSS/S. Cells were incubated with biotinylated goat anti-mouse antibody (1:300) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in H/HBSS/S for 30 min followed by three washes with H/HBSS/S and a 30-min incubation in Vectastain ABC-AP reagent (Vector Laboratories, Burlingame, CA). Cells were washed as above and incubated in Vector Red Alkaline Phosphatase substrate (Vector Laboratories) until color was visible then counterstained with Hoechst stain for 10 min at RT. Cells were viewed using a Zeiss Axiophot epifluorescence microscope with a Photometrics Quantix cooled digital camera (Tuscon, AZ) and QImaging Micropublishing was used to capture the image. A TRITC filter was used for visualizing DEN antigen, an FITC filter for the fluoresceinated DScr P-PMO, and a UV filter for viewing cellular nuclei. Photos were merged using Adobe Photoshop (San Jose, CA).

### *Quantitative real-time RT-PCR*

Cells were harvested at the times indicated using 500  $\mu$ l of TRIZOL (Invitrogen) per well. Samples were kept at  $-80^{\circ}\text{C}$  until RNA was extracted according to the manufacturer's protocol. Total RNA was resuspended in 200  $\mu$ l RNase-free  $\text{ddH}_2\text{O}$  and stored at  $-80^{\circ}\text{C}$ . Quantitative real-time RT-PCR was performed using TRIZOL-extracted total RNA samples and LUX Fluorogenic Primers (Invitrogen). For quantifying the amount of DEN RNA, a sequence within NS1 was amplified using a FAM-labeled forward primer (5'-CAC AAA CCA TGA AGA GGG CAT TTG [FAM]G-3') and an unlabeled reverse primer (5'-TTT GTT TCC ACA TCA GAT TCT CCA-3'). For quantifying reporter mRNAs containing LUC, the target sequence near the 3' end of LUC was amplified as previously described (Holden and Harris, 2004). As an internal control, LUX primers specific for mouse succinate dehydrogenase complex subunit A flavoprotein (SDHA) with a FAM label or  $\beta$ -actin with a JOE label (Invitrogen) were used. SuperScript II Platinum One-Step Quantitative RT-PCR System (Invitrogen) and an ABI PRISM 7300 (Applied BioSystems, Foster City, CA) were used according to the manufacturers' instructions. A standard curve was generated by diluting a mixture of total BHK mRNA and either pD2/IC RNA (gift of R. Kinney, CDC) or LUC mRNA that was transcribed in vitro as described below.

### *Western blot analysis*

Cells were harvested from 12-well plates 24 hpi using HBSS/E/H, washed twice in PBS, and lysed in 60  $\mu$ l of freshly made lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.01% SDS, 1% NP40, 1 mM DTT, and aprotinin) chilled on ice for 20 min. Cellular debris was pelleted by centrifugation (this step had no impact on the amount of NS1 protein present in the cell lysate [A.-M. Helt and E. Harris, unpublished data]), and the protein concentration in the supernatant was determined. Thirty micrograms of total protein were mixed with loading buffer

and boiled for 5 min alongside a biotinylated ladder (Cell Signaling Technology, Beverly, MA) and a Dual Color Precision Plus Protein Standard (Bio-Rad Laboratories, Hercules, CA) before electrophoresis in an SDS-polyacrylamide gel (10%). Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA), which was then blocked in PBS containing 5% nonfat dry milk and 0.1% Tween-20 (PBS-TM) overnight at 4 °C. Primary antibodies to DEN NS1 (a 1:1 mixture of 8H7.C10 and 2E9.G4) (P.R. Beatty, and E. Harris, unpublished data) were diluted 1:7 in PBS-TM and incubated with the blot for 2 h at RT. Both peroxidase-conjugated AffiniPure rabbit anti-mouse IgG and IgM antibodies (1:10,000) (Jackson ImmunoResearch Laboratories, Inc.) and anti-biotin HRP-conjugated secondary antibodies (1:2000) (Cell Signaling Technology) were used along with the Super-Signal West Dura Extended Duration substrate (Pierce, Rockford, IL), and image analysis was performed using a ChemiDOC system (Bio-Rad Laboratories, Hercules, CA). The blot was subsequently washed and re-probed with an HRP-conjugated monoclonal anti-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1–2 h followed by the West Dura substrate. The limit of detection of the anti-NS1 Western blot was determined by analyzing serial dilutions of 16681-infected cell lysates in a single blot.

#### Replicon cloning

A panel of sub-genomic DEN2 replicons was constructed by modification of a molecular clone of the DEN2 strain 16681, using an approach described initially by Varnavski and Khromykh (1999). Starting material for the work was a molecular clone in which the full-length genomic RNA of the 16681 DEN2 strain was positioned under the transcriptional control of the T7 promoter (pD2/IC-30P; referred to as pD2/IC) (Kinney et al., 1997), generously provided by R. Kinney (Center for Disease Control and Prevention, Fort Collins, CO). The firefly luciferase gene was placed after the first 72 nt of C followed by the foot and mouth disease virus 2A self-cleaving protease (FMDV 2Apro), which was included to cleave the luciferase protein away from the downstream NS proteins (Ryan and Drew, 1994). FMDV 2Apro was fused to the last 90 nt of the envelope gene (E), which contains the leader sequence required for proper topology of the remaining viral polyprotein in the ER during translation (Bray et al., 1989; Falgout et al., 1989). All of the non-structural genes and the DEN2 3'UTR were included downstream of E. The antisense hepatitis delta virus ribozyme (HDVr) sequence was inserted adjacent to the DEN2 3'UTR to cleave the in-vitro-transcribed RNA, generating the correct 3' end (Perrotta and Been, 1990).

Two classes of replicons were constructed under the transcriptional control of either the T7 or CMV promoters. While the pDENrep-FH vector used in these studies was designed downstream of the T7 promoter, it shared many common cloning intermediates with CMV promoter (“DNA-launched”) replicons that will be described elsewhere (T.C. Pierson and E. Harris, unpublished data). First, the entire 5' end of the pD2/IC molecular clone was replaced with sequence

encoding a sub-genomic DEN2 RNA that lacked the majority of the structural genes (residues 26–745), positioned downstream of the CMV promoter/enhancer. To do this in the smallest number of steps, overlapping PCR was performed to fuse a fragment of a DNA-launched sub-genomic WNV replicon encoding the CMV promoter, FMDV 2Apro, and a novel *MluI* site within the DEN ORF (T.C. Pierson, M. Sanchez, B.A. Puffer, A.A. Ahmed, B. Geiss, L.E. Valentine, L.A. Altamura, M.S. Diamond, and R.W. Doms, submitted for publication) followed by insertion into pD2/IC using unique *SacI* and *MluI* sites. Importantly, the *SacI* and *MluI* sites originally present in pD2/IC were destroyed during this step, allowing the replacement of WNV sequence (the 5'UTR and 20 aa of C) with sequence encoding the DEN2 5'UTR and 24 residues of C using a single PCR fragment and the novel *SacI* and *MluI* sites. The insertion of the HDVr was also introduced using overlap extension PCR and unique *AvrII* and *ClaI* sites present in the vector backbone. Subsequently, the firefly luciferase reporter gene was inserted into the unique *MluI* site located just downstream of the FMDV 2Apro (creating pDIIrep-fLuc). Finally, a fragment containing T7-DEN 5'UTR-luciferase was re-introduced into pDIIrep-fLuc using unique *SphI* and *KphI* sites (located in luciferase and the vector backbone, respectively) to create pDENrep-FH.

#### In vitro transcription and RNA transfection

pDENrep-FH was linearized with *PstI*. pGL3-5'DEN-C72-LUC-3'DEN (Holden and Harris, 2004) was linearized with *XbaI*, leaving 4 nt of vector sequence at the 3' end. After digestion, plasmid DNA was phenol-chloroform-extracted, precipitated with sodium acetate, and resuspended to approximately 1 µg/µl. 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) and was incubated at 30 °C (replicon) or 37 °C (reporter). Cap analog m<sup>7</sup>GpppA (NEB) was included in the reaction to incorporate a 5' cap structure into the mRNA. 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 using a spectrophotometer (Ultrospec 1000, Pharmacia Biotech, England) and stored at –80 °C.

For RNA transfection experiments, BHK cells were seeded ( $1 \times 10^5$  cells per well) in 12-well plates and incubated overnight. Cells were washed with VP-SFM before 2.5 µM P-PMO in 1 ml VP-SFM was added to each well. Cells were returned to the incubator for 3 h and then washed twice with VP-SFM. LipofectAMINE2000 (Invitrogen) was mixed with either 4 µg of the DEN2 reporter mRNA or 0.5 µg of DEN2 reporter replicon RNA, and cells were transfected according to the manufacturer's instructions. Transfection was stopped by washing the cells 2 h after addition of the RNA–LipofectAMINE2000 mixture. Mycophenolic acid (3 µM) (Sigma) or the P-PMOs in VP-SFM were added to each well, and, after incubation at 37 °C for 4 h, 20 µl of FBS was added to each well. Cells were harvested at the times indicated in the figure

legends. Duplicate wells were lysed for luminometry using Cell Culture Lysis Reagent (CCLR) (Promega), and two other wells were treated with TRIZOL (Invitrogen) to extract total RNA. All cell lysates and RNA samples were stored at  $-80^{\circ}\text{C}$  until processed. To perform luciferase assays, 10  $\mu\text{l}$  of lysate was mixed with 50  $\mu\text{l}$  Luciferase Assay Reagent (Promega). The samples, diluted 1:10 in CCLR if necessary, were analyzed using a TD20/20 luminometer (Turner Designs, Sunnyvale, CA) with a 3-s delay and a 15-s read.

#### Statistical analysis and mRNA half-life calculations

Standard deviation, standard deviation of the mean, and Student's *t* test were determined using Microsoft Excel for all experiments, which were repeated at least three times.

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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.2005.08.034.

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