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Inhibition of Dengue Virus Serotypes 1 to 4 in Vero Cell Cultures with Morpholino Oligomers

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Five dengue (DEN) virus-specific $R_5F_2R_4$ peptide-conjugated phosphorodiamidate morpholino oligomers (P4-PMOs) were evaluated for their ability to inhibit replication of DEN virus serotype 2 (DEN-2 virus) in mammalian cell culture. Initial growth curves of DEN-2 virus 16681 were obtained in Vero cells incubated with 20 μM P4-PMO compounds. At 6 days after infection, a P4-PMO targeting the 3′-terminal nucleotides of the DEN-2 virus genome and a random-sequence P4-PMO showed relatively little suppression of DEN-2 virus titer (0.1 and 0.9 log_{10}, respectively). P4-PMOs targeting the AUG translation start site region of the single open reading frame and the 5′ cyclization sequence region had moderate activity, generating 1.6- and 1.8-log_{10} reductions. Two P4-PMO compounds, 5′SL and 3′CS (targeting the 5′-terminal nucleotides and the 3′ cyclization sequence region, respectively), were highly efficacious, each reducing the viral titer by greater than 5.7 log_{10} compared to controls at 6 days after infection with DEN-2 virus. Further experiments showed that 5′SL and 3′CS inhibited DEN-2 virus replication in a dose-dependent and sequence-specific manner. Treatment with 10 μM 3′CS reduced the titers of all four DEN virus serotypes, i.e., DEN-1 (strain 16007), DEN-2 (16681), DEN-3 (16562), and DEN-4 (1036) viruses by over 4 log_{10}, in most cases to below detectable limits. The extent of 3′CS efficacy was affected by the timing of compound application in relation to viral infection of the cells. The 5′SL and 3′CS P4-PMOs did not suppress the replication of West Nile virus NY99 in Vero cells. These data indicate that further evaluation of the 5′SL and 3′CS compounds as potential DEN virus therapeutics is warranted.

Dengue fever-dengue hemorrhagic fever (DF-DHF) is a complex of clinical syndromes caused by dengue (DEN) viruses of the genus *Flavivirus* (family *Flaviviridae*). DF-DHF has become a major global health problem over the past 25 years, producing more human illness than any other insect-transmitted viral disease (7). The geographic distribution of DEN viruses, their mosquito vectors (primarily *Aedes aegypti*), and the resulting disease burden continues to increase. The World Health Organization estimates that over 50 million new human infections occur annually. DF-DHF has become a leading cause of hospitalization and death among children in Southeast Asia (31), and the incidences of infection and disease are sharply rising in the Americas (8). There is currently no commercially available vaccine, chemoprophylactic, or effective therapeutic. Medical supportive care is the recommended primary treatment modality to improve the condition of severely infected patients (20).

The DEN virus genome is an approximately 10.7-kb single-stranded RNA of positive polarity with a 5′ cap and lacking a 3′ poly(A) tail. DEN virus genomic RNA has a single long open reading frame (ORF) encoding three structural and seven nonstructural proteins. The mature DEN virus proteins are derived by co- or posttranslational cleavage of the polyprotein encoded by the ORF. The ORF is flanked by 5′ and 3′ untranslated regions (5′ and 3′ UTRs) that have various functions in the initiation and regulation of viral translation, replication, and assembly (21, 24, 34).

Serologic and molecular analyses have defined four antigenically distinct serotypes of DEN virus (DEN virus serotype 1 [DEN-1 virus], DEN-2 virus, DEN-3 virus, and DEN-4 virus), which exhibit overall amino acid sequence identity of approximately 70% (12). Each serotype, in turn, contains several genotypic strains. The four DEN virus serotypes have pantropical distributions and cause similar spectra of clinical conditions (7). Persons who have experienced a single DEN virus infection generally develop a long-lasting immunity to DEN virus strains of the same serotype. However, subsequent infection with a different DEN virus serotype has a likelihood of leading to severe disease, at least in part through an immunologic process known as antibody-dependent enhancement (10, 18, 27). It is essential that a prospective vaccine and highly desirable that a prospective therapeutic be effective against all four DEN virus serotypes. Whether or not a safe and efficacious tetravalent DEN virus vaccine becomes available in the near future, there remains a critical need to develop effective therapeutics to treat patients suffering from severe DEN virus infection.

Antisense compounds of various structural types have been successfully used to affect gene expression in several viral pathogens of humans (23). An antisense therapeutic for cytomegalovirus retinitis that targets the cytomegalovirus immediate-early gene 2 product has received approval from the Food and Drug Administration (25), and a number of oligonucleotide compounds with various structural chemistries are in clin-

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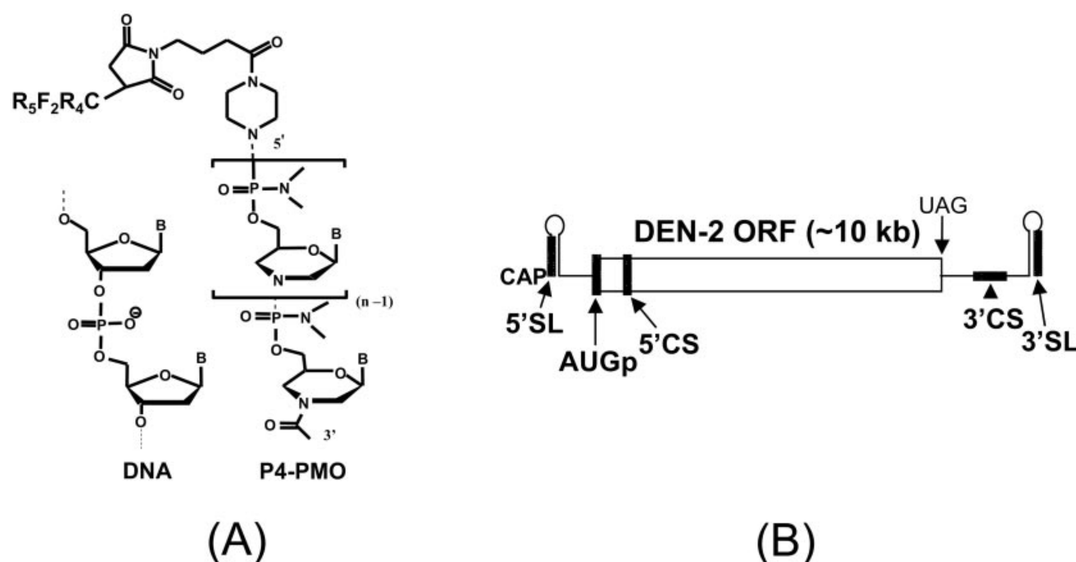

FIG. 1. P4-PMO structure and DEN virus genomic targets. (A) The deoxyribose rings and phosphodiester intersubunit linkages of DNA are replaced by morpholine rings and phosphorodiamidate intersubunit linkages in P4-PMO. The arginine-rich peptide $R_5F_2R_4C$ is linked to the 5′ end of the PMO by a noncleavable linker. B represents the base A, G, C, or T. (B) Schematic diagram of antisense P4-PMO target locations in the DEN-2 virus genome. Relative locations of viral target sense sequences are indicated by dark bars. The stem-loop structures in the 5′ and 3′ untranslated regions are stylized and simplified. Abbreviated compound names and target regions are as follows: 5′SL, 5′ terminus; AUGp, initiation site for the translated polyprotein; 5′CS, 5′ cyclization sequence; 3′CS, 3′ cyclization sequence; 3′SL, 3′ terminus. The UAG termination codon of the translated polyprotein is also shown.

ical trials (23). Delivery of antisense compounds to their intended RNA targets within relevant cells remains a major hurdle to the clinical feasibility of the technology, and a number of transporters and delivery-enhancing strategies have been developed (4). An antisense study using phophorothioate oligonucleotides containing C-5 propyne-substituted pyrimidines microinjected into LLC-MK_2 cells, which were subsequently infected with DEN-2 virus, has been described (36). That study documented a 50 to 75% reduction in viral titer with compounds targeted both to positive-strand sequences immediately downstream from the AUG start site of the translated polyprotein and to the DEN virus 3′ UTR in two regions separate from those targeted in the study described here.

Phosphorodiamidate morpholino oligomers (PMOs) are a class of antisense compounds that contain purine or pyrimidine bases attached to a backbone composed of six-member morpholine rings joined by phosphorodiamidate intersubunit linkages and are typically synthesized to about 20 subunits in length (41). PMOs are water soluble, are nuclease resistant (11), and bind to RNA by Watson-Crick base pairing (40, 41). A PMO-RNA duplex does not form a substrate for RNase H (38), and therefore PMOs are considered a "steric-blocker" antisense structural type. The mechanism of action of these compounds is through stable and sequence-specific duplexing with RNA, thereby obstructing access of biomolecules to a particular sequence of RNA. PMOs have demonstrated effective and specific suppression of the replication of several RNA viruses (30, 39). Conjugation of arginine-rich peptides to PMOs has been shown to greatly increase PMO cellular uptake and inhibitory efficacy against specific targets in cell culture systems compared to PMOs either lacking a peptide conjugate or conjugated with various other peptides (28, 30). Those researchers achieved nontoxic cellular entry and subsequent antisense efficacy of arginine-rich peptide-PMOs by incubation under standard cell culture conditions.

The purpose of this study was to evaluate the efficacies and specificities of five arginine-rich peptide-PMOs in inhibiting the productive replication of DEN-1 to -4 viruses in Vero cells in culture. The five compounds were designed to target sequence elements in the positive strand of DEN-2 virus RNA that have been previously implicated as important for flavivirus translation and/or replication.

MATERIALS AND METHODS

Cells and viruses. Vero cells and wild-type DEN-1 16007, DEN-3 16562, and DEN-4 1036 viruses were available in the collections at the Centers for Disease Control and Prevention, Fort Collins, Colo. The derivation of D2/IC-30P-A (DEN-2) virus from an infectious cDNA clone of wild-type DEN-2 16681 virus has been described previously (16). The D2/IC-30P-A virus is essentially identical to its progenitor 16681 virus in genome sequence, in vitro replication phenotype, and mouse neurovirulence (2, 16). Confluent monolayers of Vero cells were grown in Iscove's medium (HyClone, Logan, Utah) supplemented with 9% heat-inactivated fetal bovine serum (FBS) (HyClone), sodium bicarbonate (0.75 g/liter), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) (indicated as Iscove–9% FBS medium) in 12-well plates at 37°C and 5% CO_2. Media containing 4.7% FBS (Iscove–4.7% FBS) or lacking FBS (Iscove–0% FBS) were also used in this study.

PMO design and synthesis. Each PMO was synthesized with the arginine-rich peptide NH_2-RRRRRFFRRRRC-$CONH_2$ ($R_5F_2R_4$) covalently linked to its 5′ end (Fig. 1A). The procedures for the synthesis, conjugation, purification, and analysis of $R_5F_2R_4$-PMO (P4-PMO) compounds were identical to those recently described for R_9F_2-PMO (28). P4-PMO compounds of 18 to 20 subunits in length were designed to target, by complementary base pairing, five sequence elements in the positive-strand DEN-2 virus RNA that have been identified as important in flavivirus translation and/or replication (3, 14, 15, 21, 24, 42, 45). The P4-PMO sequences, designations, and target locations are described in Table 1 and Fig. 1B. A 20-mer P4-PMO with a randomly generated sequence having 50% GC content was prepared to control for non-sequence-specific ac-

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TABLE 1. P4-PMO sequences (3′→5′) and target flavivirus genomic sequences (5′→3′)

P4-PMO	DEN-2 virus target region	P4-PMO and target sequence[a]	P4-PMO or virus
5′SL	5′ terminus	3′-[TCAACAATCAGATGCACCTG]-5′	P4-PMO
		5′-1-[AGUUGUUAGUCUACGUGGAC]-20-3′	DEN-2
		5′-1-[********************]-20-3′	DEN-1
		5′-1-[********************]-20-3′	DEN-3
		5′-1-[************GU******]-20-3′	DEN-4
		5′-1-[***A***C*C**GU***AG*]-20-3′	WN
AUGp[b]	AUG of ORF	3′-[GTCTAGAGACTACTTATTGG]-5′	P4-PMO
		5′-87-[CAGAUCUCUGAUGAAUAACC]-106-3′	DEN-2
5′CS	5′ cyclization	3′-[GGAAAGTTATACGACTTTGC]-5′	P4-PMO
		5′-130-[CCUUUCAAUAUGCUGAAACG]-149-3′	DEN-2
3′CS	3′ cyclization	3′-[GTCGTATAACTG-CGACCC]-5′	P4-PMO
		5′-10618-[CAGCAUAUUGAC-GCUGGG]-10635-3′	DEN-2
		5′-10630-[************-******]-10647-3′	DEN-1
		5′-10591-[************-******]-10608-3′	DEN-3
		5′-10543-[************-******]-10560-3′	DEN-4
		5′-10922-[************AC*****]-10940-3′	WN
3′SL	3′ terminus	3′-[GACAACTTAGTTGTCCAAGA]-5′	P4-PMO
		5′-10704-[CUGUUGAAUCAACAGGUUCU]-10723-3′	DEN-2
DScr[c]	Control	3′-[ACTCCATCGTTCAGCTCTGA]-5′	P4-PMO

[a] The antisense sequence of each P4-PMO is written 3′ to 5′. The viral genomic target sense sequences are written 5′ to 3′. Asterisks in the DEN-1, -3, -4, and WN virus sense sequences indicate nucleotide sequence identity with the DEN-2 virus target sense sequence. The dashes in the 3′CS P4-PMO and viral target sequences indicate a deletion, relative to the target sense sequence of WN virus. The underlined positions in the viral 3′CS sense sequences indicate the 3′ UTR nucleotides that hybridize with the corresponding 5′CS of DEN-2 (genome nucleotide positions 134 to 144), DEN-1 (133 to 143), DEN-3 (132 to 142), DEN-4 (136 to 146), and WN (135 to 146) viruses. The 5′ and 3′ genomic nucleotide positions are shown for each viral target sense sequence.

[b] AUGp, P4-PMO directed against the sequence region containing the AUG initiation codon for the single genomic ORF.

[c] DScr, non-virus-specific control P4-PMO synthesized with randomly generated sequence.

tivity of the P4-PMO chemistry. Antisense and negative control P4-PMO sequences were screened with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against all primate mRNA sequences, and the random-sequence compound additionally was screened against all flavivirus sequences, to preclude unintentional gene-silencing effects.

Viral growth curves in the presence of P4-PMO compounds. The lyophilized P4-PMOs were dissolved in sterile phosphate-buffered saline (PBS) and stored at 4°C prior to use. Vero cells were seeded into 12-well plates at 5.0 to 5.5 $\log_{10}$ cells per well. Unless otherwise stated, Vero cells grown to monolayer confluency in the 12-well plates were pretreated with P4-PMO for 12 h prior to infection with DEN virus and then retreated with the same concentration of P4-PMO after inoculation and adsorption of virus in duplicate wells. Viral infection was performed by aspirating the growth medium from freshly confluent Vero cell cultures, washing the cells sheets twice with 2 ml of Iscove–0% FBS medium, and adding 100 μl of Iscove–0% FBS medium containing DEN or WN virus to deliver a multiplicity of infection (MOI) of 1.0 or 2.0 PFU/cell. Following adsorption of virus for 2 h at 37°C with 5% CO_2, the viral inocula were aspirated, the cell sheets were rinsed three times each with 2 ml of PBS, and 1.0 ml of Iscove–0% FBS medium containing the appropriate concentration of P4-PMO was added, followed by incubation of the plates at 37°C with 5% CO_2. Except where stated, the replacement media were not changed again for the duration of the growth curve experiment. Controls for these experiments included untreated cells; cells infected with DEN-1, -2, -3, -4, or WN virus (duplicate wells) in the absence of P4-PMO; and uninfected cells treated with each P4-PMO at 1 to 20 μM (single well each). At various time intervals, a 20-μl aliquot of medium was removed from each virus-infected well, diluted 1:16 or 1:32 in freezing medium (Iscove–35% FBS medium), and stored at −80°C until plaque titration. Plaque titrations were performed under agarose overlay in Vero cell monolayers grown in six-well plates as described previously (2, 26). In each viral growth curve, the sensitivity limit of plaque titration is indicated by a horizontal line at 1.9 or 2.2 $\log_{10}$ PFU/ml, which resulted from plating 200 μl of the 1:16 or 1:32 dilution of harvested virus in the first well of the six-well plate, respectively.

Cytotoxicity assay. A quantitative colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, St. Louis, Mo.) assay was used to quantify cell viability in response to treatment with P4-PMOs. Briefly, Vero cells were plated at a density of 4.2 $\log_{10}$ cells per well, in minimal essential medium containing 10% FBS, in a flat-bottom 96-well culture dish and allowed to adhere overnight. The following day, 100 μl of minimal essential medium lacking serum and containing either appropriate concentrations of P4-PMOs or water was applied in triplicate to culture wells. The cells were incubated at 37°C with 5% CO_2 for 24 h. After treatment, 10 μl of yellow MTT stock solution (5 mg/ml in 0.1 M Tris-buffered saline) was added to each well, and the cells were incubated further until purple formazan salt crystals became visible under microscopic examination. The MTT-medium solution was removed, and the cells were solubilized with 200 μl of dimethyl sulfoxide. This solution was transferred to a new 96-well plate, and the optical density of each sample was determined on a microplate spectrophotometer (VERSAmax; Molecular Devices, Sunnyvale, Calif.) at a wavelength of 540 nm by using the SOFTmax Pro program (Molecular Devices).

Virus-specific immunofluorescence in Vero cells treated with P4-PMO compounds. Wells of Lab-Tek eight-chamber slides (Nalge Nunc, Naperville, Ill.) were seeded with 4.9 $\log_{10}$ Vero cells from subconfluent stock cultures. After 24 h, the nearly confluent cell sheets were rinsed twice with Iscove–0% FBS medium, and 210 μl of the same medium containing P4-PMO was added. Following 12 h of preincubation with the P4-PMO compound, the medium was aspirated, and the cells were infected with DEN-2 or WN virus. After adsorbing for 2 h at 37°C, the virus inoculum was aspirated, the cell sheets were rinsed three times with Iscove–0% FBS medium, 210 μl of the same medium containing the preincubation concentration of P4-PMO was again added, and the cultures were incubated for 2 or 4 days at 37°C with 5% CO_2. Mock-infected cells were treated in an identical fashion except for incubation for 2 h with Iscove–0% FBS medium instead of viral inoculum. For immunofluorescence assay (IFA), the cells were fixed with acetone for 30 min at −20°C, air dried, and incubated for 1 h at 37°C with primary antibody (mouse immune ascitic fluid against DEN-2 New Guinea C virus or mouse hyperimmune ascitic fluid against WN Eg101 virus). The slides were then rinsed three times with PBS, and primary antibody binding was detected by incubation for 45 min at 37°C with fluorescein isothiocyanate-labeled goat anti-mouse antibody (Jackson Immuno Research, West Grove, Pa.). Following three PBS rinses, coverslips were mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, Ala.). Bright-field (reflected-light

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Nomarski differential interference contrast) and fluorescent cell images were captured with an Olympus BX51 microscope (with fluorescence, differential interference contrast, and phase contrast) and using MagnaFire SP software.

RESULTS

Design of five antisense P4-PMO compounds. Antisense P4-PMO compounds were designed to target highly conserved and/or critical elements of the DEN-2 virus genome. 5′SL targeted the 20 5′-terminal nucleotides of the DEN-2 virus genome (Table 1; Fig. 1B). The region of sequence adjacent to the 5′ cap is thought to be involved in assembling the 40S ribosomal subunit and translation initiation factors onto mRNA in eukaryotic cells (32). Moreover, the 5′SL target site is located in the 5′-terminal stem-loop, a structural feature conserved among flavivirus genomes (1, 42). The 5′SL target site sequence is perfectly conserved in DEN-1, -2, and -3 viruses and has two base mismatches in DEN-4 virus (Table 1).

Previous studies with PMO compounds have shown that the translation-initiator codon region is often an effective target site (6, 29, 30, 40), presumably by blocking the interaction of the initiator AUG with the Met-tRNA$_i$ in the 48S preinitiation complex, thereby obstructing the 60S ribosomal subunit from joining the preinitiation complex. AUGp was designed to hybridize with bases −10 to +7 (relative to the AUG codon) of the DEN-2 virus polyprotein ORF (Table 1; Fig. 1B). This region has relatively poor sequence conservation across DEN virus serotypes.

DEN virus, like all members of the genus *Flavivirus*, requires a long-range RNA-RNA interactive cyclization between the 5′ and 3′ genomic ends for efficient replication (9, 15, 44). P4-PMOs were designed to duplex with the 5′ cyclization motif and its flanking sequence, located in the coding region 31 to 50 bases downstream from the AUG translation start site, as well as the 3′ cyclization sequence region, located 89 to 106 bases upstream from the 3′ terminus of the viral genome (Table 1; Fig. 1B). The target sequence of 3′CS is perfectly conserved across all DEN virus serotypes. The target of 5′CS is perfectly conserved across DEN-1, -2, and -4 viruses but deviates at two positions in DEN-3 virus RNA.

The 3′SL P4-PMO was directed to the 20 3′-terminal bases of the genome (Table 1; Fig. 1B). The 3′-terminal region of DEN is predicted to fold into a configuration of secondary, and possibly tertiary, structure (24, 33–35, 37, 42) and has high but imperfect conservation across serotypes. The 3′-terminal stem-loop structure and sequence have been reported to have an essential role in flavivirus replication (43, 46).

Screening of five P4-PMO compounds against DEN-2 virus grown in Vero cells. Vero cells were grown in Iscove–9% FBS medium and then maintained in Iscove–0% FBS medium during P4-PMO treatment and viral infection. In several separate experiments, DEN-2 virus replication was equivalent in cells maintained in Iscove–4.7% FBS or Iscove–0% FBS medium (data not shown). Cells maintained in medium containing various mixtures of up to 50% Iscove–0% FBS medium and 50% PBS supported DEN-2 virus replication equally well (data not shown). These experiments demonstrated robust viral replication in the absence of FBS, a condition necessary for efficient delivery of P4-PMO compounds into cells (28, 30).

The five antisense P4-PMO compounds and the random-sequence DScr P4-PMO listed in Table 1 were compared at a single concentration of 20 μM for their ability to inhibit DEN-2 virus replication in Vero cells (Fig. 2). DEN-2 virus titers were monitored daily during days 1 to 4, and again on days 6 and 8, after infection. Figure 2 shows the viral titers in the culture medium at 6 days after infection. DEN-2 virus replicated to a mean peak titer of 7.9 $\log_{10}$ PFU/ml at day 6 after infection in the absence of P4-PMO treatment. Three of the P4-PMO compounds, targeting the AUG translation start site region (AUGp), 5′ cyclization sequence (5′CS), and 3′-terminal stem-loop (3′SL), showed 1.6 $\log_{10}$, 1.8 $\log_{10}$, and 0.1 $\log_{10}$ suppression of the corresponding DEN-2 virus mean titer, respectively, at 6 days after infection (Fig. 2). The random-sequence P4-PMO DScr compound resulted in 0.9 $\log_{10}$ inhibition. The two P4-PMO compounds targeting the 5′-terminal stem-loop (5′SL) and the 3′ cyclization sequence (3′CS) were highly efficacious, each reducing the mean titer of DEN-2 virus by greater than 5.7 $\log_{10}$ relative to the non-P4-PMO-treated DEN-2 virus-infected cultures.

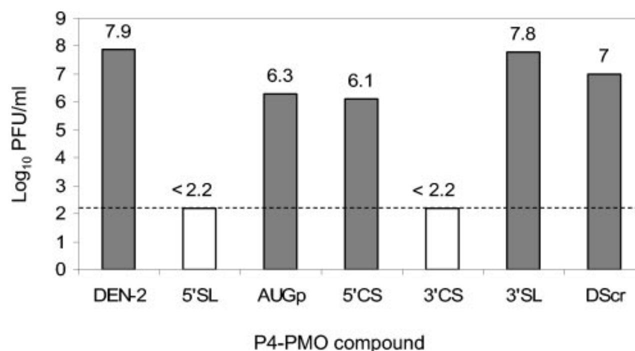

FIG. 2. DEN-2 virus replication in the presence of five virus-specific antisense P4-PMO compounds and one random-sequence P4-PMO (DScr) at 6 days after infection of Vero cells. Cultures were pretreated with 20 μM P4-PMO for 12 h before infection with DEN-2 virus at an MOI of 2.0. Incubation with 20 μM P4-PMO continued following viral adsorption. The mean $\log_{10}$ viral titers of duplicate wells at day 6 are indicated above each bar. The horizontal line at 2.2 $\log_{10}$ indicates the sensitivity limit in this experiment. Viral replication in the presence of 5′SL and 3′CS was suppressed to undetectable levels in this experiment (open bars). Abbreviated compound names and target viral genomic regions are as indicated in Fig. 1B.

At the 20 μM concentration, the P4-PMOs generated significant cytotoxicity, relative to nontreated Vero cells (data not shown). The 5′SL and AUGp P4-PMOs had the most severe effect on the Vero cells, resulting in a cytopathic effect (CPE) that was characterized by detachment of about 15 to 30% of the cells in P4-PMO-treated, uninfected wells by day 8 of incubation. Although these two P4-PMOs showed similar levels of CPE, DEN-2 virus replication remained quite robust in the AUGp-treated cells (6.3 $\log_{10}$ PFU/ml at day 6) relative to the extreme restriction of DEN-2 virus replication caused by the 5′SL P4-PMO (less than 2.2 $\log_{10}$ PFU/ml) (Fig. 2). The 5′CS P4-PMO resulted in approximately 5 to 15% detachment of cells in uninfected wells by day 8 after infection. The inhibitory effect of the 5′CS P4-PMO on DEN-2 virus replication was nearly identical to that of the AUGp P4-PMO. Although extensive rounding of cells occurred in uninfected Vero cell sheets treated with the 3′CS P4-PMO compound, the cell

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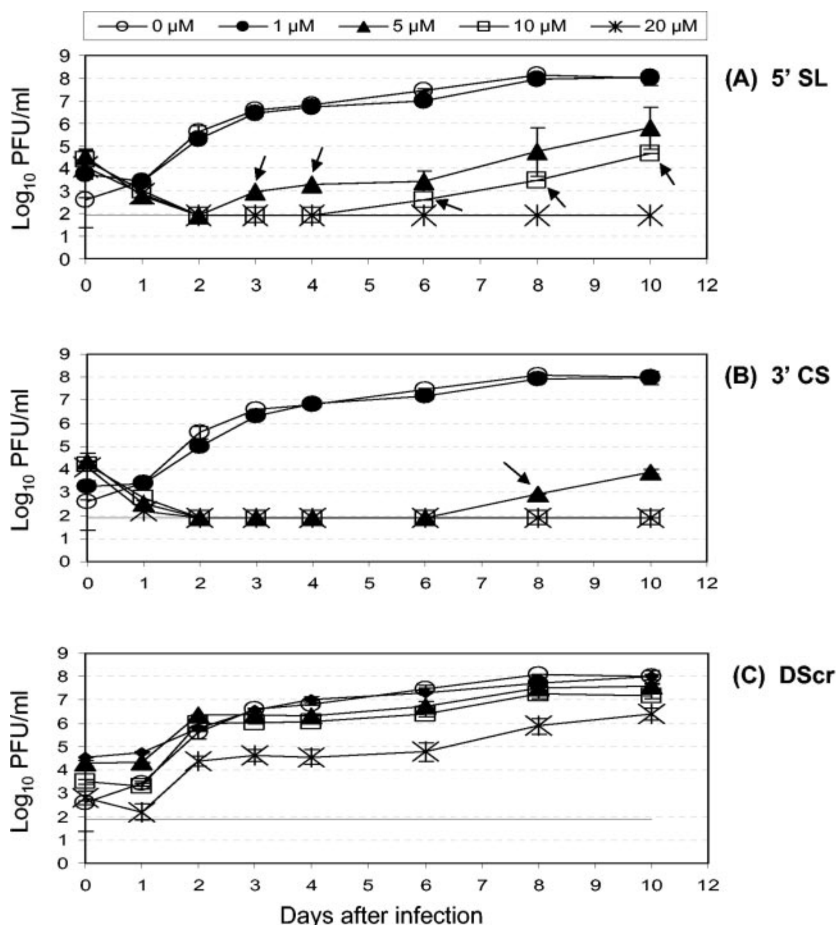

FIG. 3. Growth curves of DEN-2 virus in Vero cells in the presence of various concentrations of P4-PMO compounds. (A) 5′SL (targeting the DEN-2 virus genomic 5′ terminus); (B) 3′CS (3′ cyclization sequence); (C) random-sequence control DScr. Cultures were pretreated with the indicated concentration of each P4-PMO for 12 h before infection with DEN-2 virus at an MOI of 1.0. Cultures continued to be incubated in the presence of the same concentration of each P4-PMO following viral adsorption. Each graph point is the mean DEN-2 virus $\log_{10}$ titer (± high and low titer for each individual replicate) determined for duplicate Vero culture wells. The minimum detectable threshold of 80 PFU/ml is indicated by the horizontal line at 1.9 $\log_{10}$. The arrows in (A) and (B) indicate the discrete DEN-2 virus titer from a single replicate well; the viral titer in the second replicate well was below the threshold of detection of 1.9 $\log_{10}$ PFU/ml. Therefore, accurate mean viral titers were not obtainable for these particular graph points.

sheets were observed to remain essentially intact at day 8 of incubation. Despite the relatively low level of CPE induced by the 3′CS P4-PMO, DEN-2 virus replication was suppressed by greater than 5.7 $\log_{10}$, compared to DEN-2 virus-infected cells lacking P4-PMO treatment, at 6 days after infection (Fig. 2). The 3′SL and DScr control P4-PMOs caused somewhat less CPE than did the 3′CS P4-PMO, and the effect of these two P4-PMOs on DEN-2 virus replication was low, showing 0.1 and 0.9 $\log_{10}$ reductions, respectively, in mean viral titer at day 6 (Fig. 2). Although there appeared to be some connection between the CPE level induced by the various P4-PMOs and the resulting reduction in mean DEN-2 virus titer, a clear P4-PMO-specific anti-DEN-2 virus effect with the 5′SL and 3′CS compounds was evident.

Dose-response studies with 5′SL and 3′CS P4-PMO compounds against DEN-2 virus. The two compounds that inhibited DEN-2 virus replication most significantly, 5′SL and 3′CS, and the random-sequence control DScr P4-PMO were selected for further analyses in this study. The effective P4-PMO dose range was determined by incubating DEN-2 virus in the presence of 1, 5, 10, and 20 μM concentrations of each P4-PMO (Fig. 3). Except for using an MOI of 1.0 in this dose-response experiment, the conditions were identical to those described for the previous experiment (Fig. 2), including preincubation of Vero cells with the appropriate P4-PMO for 12 h prior to DEN-2 virus infection and continuing P4-PMO treatment after the 2-h viral adsorption period. DEN-2 virus titers were determined in duplicate wells at day 0 (4 h after infection) and at additional times up to 10 days after infection (Fig. 3). All three P4-PMOs were ineffective at the 1 μM concentration, with the DEN-2 virus growth curves being indistinguishable from the growth curve of DEN-2 virus in non-P4-PMO-treated cells. Dramatic effects on DEN-2 virus replication were observed at 5′SL and 3′CS P4-PMO concentrations at or above 5 μM (Fig. 3A and B). The control DScr P4-PMO showed only a minor suppressive effect, less than 1.0 $\log_{10}$ inhibition of DEN-2 virus replication, at the 5 and 10 μM concentrations (Fig. 3C). Both the 5′SL and 3′CS P4-PMOs suppressed DEN-2 virus replica-

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tion by greater than 3 $\log_{10}$ during days 2 to 8 after infection (Fig. 3A and B). The 5 and 10 μM concentrations of the 3′CS compound were particularly effective, suppressing mean DEN-2 virus titers to below the minimum detectable threshold of 80 (1.9 $\log_{10}$) PFU/ml for at least 6 and 10 days, respectively, after viral infection (Fig. 3B). The DScr control P4-PMO showed a significant inhibitory effect on DEN-2 virus replication, up to a 2.7 $\log_{10}$ reduction in titer at 6 days after infection (MOI of 1.0), at the 20 μM concentration in this experiment (Fig. 3C), which was significantly greater than the 6-day 0.9 $\log_{10}$ reduction in titer observed for this concentration of DScr in the previous experiment (MOI of 2.0) (Fig. 2). At the time of DEN-2 virus infection, the cell density in non-P4-PMO-pretreated wells was about 15% lower than that in the previous experiment, which may have contributed to the observed differences in the 20 μM DScr effect. Significant levels of virus remained at 4 h after infection (day zero time point in the graphs of growth curves), despite washing the cells sheets three times after the 2-h viral adsorption period. Given the high suppressive efficacy of the 5′SL and 3′CS P4-PMO treatments at concentration of 5 μM or higher, it is likely that the day-1 DEN-2 virus titers observed for the latter treatments represented lingering virus inoculum (Fig. 3).

The 10 and 20 μM concentrations of the 3′CS P4-PMO and the 20 μM concentration of the 5′SL P4-PMO reduced mean DEN-2 virus titers to below the detection threshold during days 2 to 10 after infection. These P4-PMO effects constituted greater than a 6 $\log_{10}$ reduction in DEN-2 virus titers at days 8 and 10 after infection (Fig. 3A and B). The increase in DEN-2 virus titers between 6 and 10 days after infection in the presence of 5 μM 3′CS and 10 μM 5′SL suggested that low rates of viral replication occurred or that the DEN-2 virus genome persisted in at least a portion of the cells until conditions permitted viral replication.

Vero cell viability following 24 h of treatment with the various concentrations of the 5′SL, 3′CS, and DScr P4-PMOs was quantitatively assessed by a colorimetric MTT cell viability assay. The compounds showed dose-dependent 24-h cytotoxic effects on Vero cells (Fig. 4). The observation that the cytotoxic effects of the 5′SL and 3′CS P4-PMOs were less pronounced than those of the DScr control P4-PMO supports the conclusion that the severe suppression of DEN-2 virus titer induced by the 5′SL and 3′CS P4-PMOs was a sequence-specific antisense effect.

Effect of 5′SL and 3′CS P4-PMO compounds against DEN-1, DEN-3, and DEN-4 viruses. The 5′SL and 3′CS P4-PMOs demonstrated highly effective inhibition of DEN-2 virus replication in Vero cells. Nucleotide sequence alignments showed that these two P4-PMO compounds would be expected to have inhibitory activities against DEN-1, DEN-3, and DEN-4 viruses as well (Table 1). The 5′SL, 3′CS, and control DScr P4-PMOs were tested at the 10 μM concentration for their ability to inhibit the replication of DEN-1, DEN-3, and DEN-4 viruses (Fig. 5). These results can be compared directly to the parallel DEN-2 virus studies reported in Fig. 3. DEN-1, -3, and -4 viruses replicated to mean peak titers of 8.6, 8.1, and 8.5 $\log_{10}$ PFU/ml, respectively, at days 3 to 6 after infection at an MOI of 1.0 (Fig. 5A to C). All three heterologous DEN virus serotypes replicated more quickly than did the DEN-2 virus, which showed a mean peak titer of about 8.0 $\log_{10}$ PFU/ml at days 6 to 8 after infection (Fig. 2 and 3).

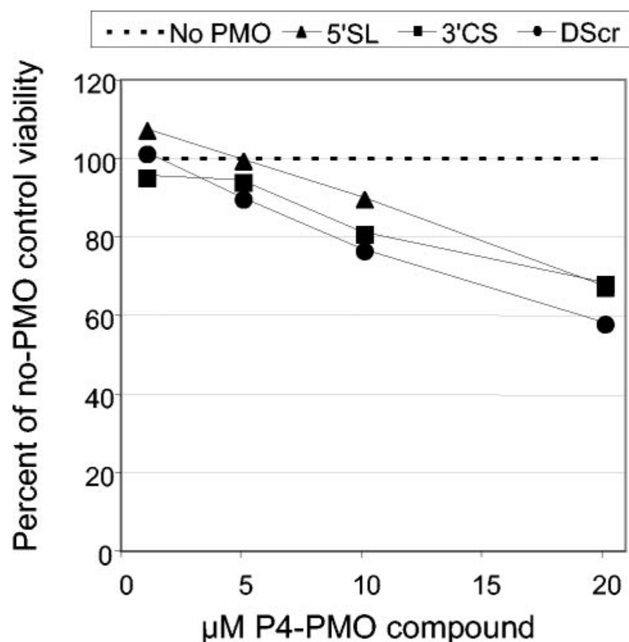

FIG. 4. Quantitative colorimetric MTT assay used to quantify Vero cell viability in response to treatment with various concentrations of the P4-PMOs 5′SL, 3′CS, and random-sequence DScr. Assays were performed in triplicate at 24 h following initiation of P4-PMO treatment. The viability of P4-PMO-treated cells is plotted as the percentage of that of non-P4-PMO-treated cells (100%).

At a concentration of 10 μM, the 3′CS P4-PMO induced extreme decreases in DEN-1, -3, and -4 virus titers (Fig. 5A to C). Viral titers were inhibited by the 3′CS P4-PMO to below the threshold detection level (1.9 $\log_{10}$) during days 2 to 8, days 2 to 4, and days 2 to 3 for DEN-1, DEN-3, and DEN-4 viruses, respectively. Although the initial suppression of viral titers was very strong, the DEN-1, -3, and -4 virus titers rebounded to approximately 3, 4, and 5 $\log_{10}$ PFU/ml by day 10, respectively (Fig. 5A to C).

As with DEN-2 virus (Fig. 3A), DEN-1 virus titers in the presence of 10 μM 5′SL were well below control levels throughout the 10-day experiment (Fig. 5A). Despite possessing a target sequence identical to those of DEN-1 and DEN-2 viruses, DEN-3 virus replication was less effectively inhibited by 10 μM 5′SL P4-PMO (Fig. 5B). The 5′SL P4-PMO inhibited DEN-3 virus titers by 3 $\log_{10}$ to greater than 4 $\log_{10}$ during early replication at days 2 to 6 after infection, after which time the mean DEN-3 virus titers in the P4-PMO-treated cells rapidly reached equivalence with the mean DEN-3 virus titers in non-P4-PMO-treated cells (Fig. 5B). The inhibitory effect of 5′SL was even less pronounced against DEN-4 virus, which exhibited a 2- to 3-$\log_{10}$ reduction in mean viral titer at days 2 to 6 after infection in the presence of 10 μM 5′SL. This reduced effect was likely due, at least in part, to the two-base mismatch between the 5′SL antisense sequence and its target sense sequence in the DEN-4 virus RNA genome (Table 1).

The 10 μM negative control DScr P4-PMO inhibited DEN-1, DEN-3, and DEN-4 virus titers by 0.7 to 2.1 $\log_{10}$

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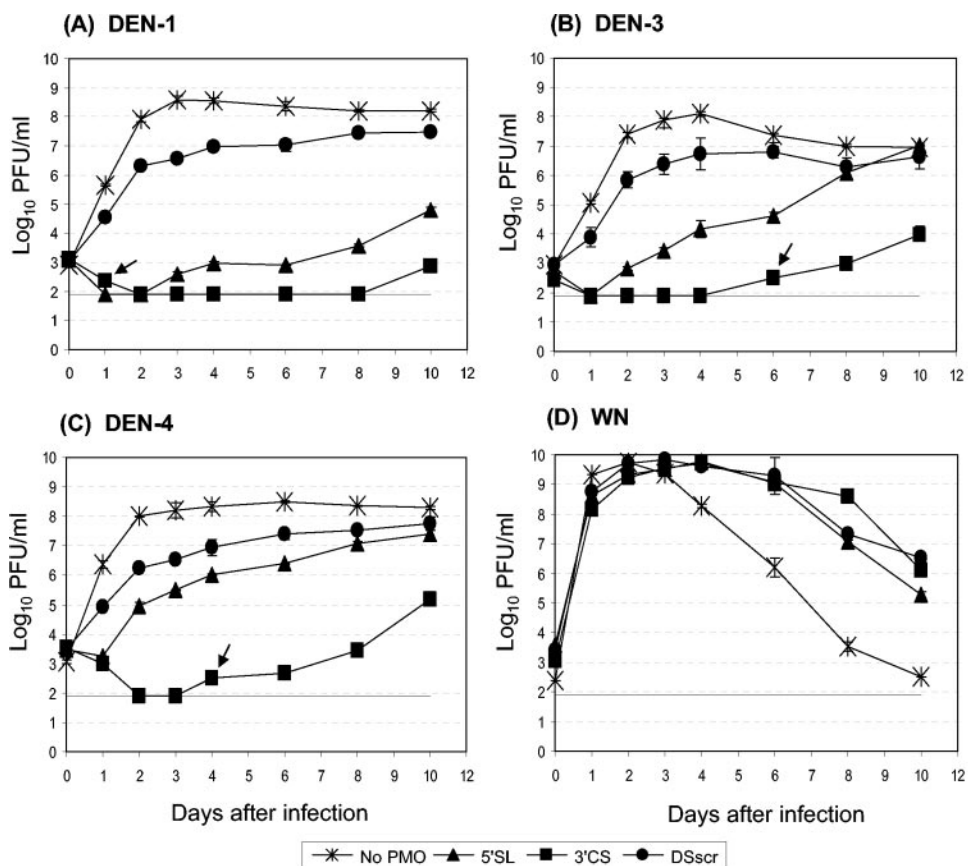

FIG. 5. Growth curves of (A) DEN-1, (B) DEN-3, (C) DEN-4, and (D) WN viruses in Vero cells in the presence of a 10 μM concentration of the P4-PMOs 5′SL (targeting the DEN-2 virus genomic 5′ terminus), 3′CS (3′ cyclization sequence), and random-sequence DScr. The MOI was 1.0 for each virus. Treatment and data presentation, including arrows, are as in Fig. 3, except that only 10 μM P4-PMO was used.

PFU/ml, relative to non-P4-PMO-treated virus-infected Vero cells, between days 2 and 6 after viral infection (Fig. 5A to C).

Effect of 5′SL and 3′CS P4-PMO compounds against WN virus. The 5′SL, 3′CS, and control DScr P4-PMOs were also tested at the 10 μM concentration for their ability to inhibit the replication of the more distantly related WN flavivirus (Fig. 5D). This WN virus experiment was performed at the same time as the previous two experiments (P4-PMO dose response against DEN-2 virus and 10 μM P4-PMO activities against DEN-1, -3, and -4 viruses), and all experimental conditions and reagent solutions were identical. The WN NY99 virus exhibited very robust replication, reaching a mean peak titer of 9.9 $\log_{10}$ PFU/ml by day 3 after infection at an MOI of 1.0. Unlike the four DEN virus serotypes, which produced little CPE during the first several days after infection, the WN virus was rapidly cytopathic and resulted in clearing of greater than 50% of the substrate plastic by day 8 after infection in non-P4-PMO-treated Vero cells. The rapid decline in mean WN virus titer during days 4 to 10 after infection of non-P4-PMO-treated Vero cells was almost certainly caused by the severe cytopathic effects of this virus.

As might be expected from the degree of nucleotide mismatch between the P4-PMO antisense sequences and the target sense sequences of the WN virus genome (Table 1), all three P4-PMOs failed to inhibit WN virus replication to an appreciable extent (Fig. 5D). Interestingly, all three 10 μM P4-PMO treatments decreased the rate of decay of the mean WN virus titer, relative to that observed in non-PMO-treated, WN virus-infected cells, at days 4 to 10 after infection (Fig. 5D). Comparative CPE levels did not show significant correlations with this observed P4-PMO effect on WN virus replication at 6 to 10 days after infection, although the 3′CS-treated, WN virus-infected cells exhibited slightly decreased CPE relative to the other WN virus-infected cultures at days 2 to 8 after infection (data not shown).

We observed very high, uninhibited mean WN virus titers (Fig. 5D), as well as the most severe inhibition of DEN virus replication (Fig. 2, 3A and B, and 5A to C), during the early days (days 1 to 4) after viral infection of Vero cells treated with the 5′SL or 3′CS P4-PMO compound. Because viral propagation is expected to be diminished by cytopathic events, we considered these results an affirmation of the 5′SL and 3′CS P4-PMO virus-specific inhibitory effect, particularly at the 5 and 10 μM concentrations, against DEN virus replication in Vero cells.

Effect of time of addition and duration of 3′CS P4-PMO treatment on DEN-2 virus. Having established the potent anti-DEN virus activity of 3′CS in trials in which Vero cells were pretreated with the compound for 12 h before virus inoculation and treated again with P4-PMO following the 2-h viral adsorp-

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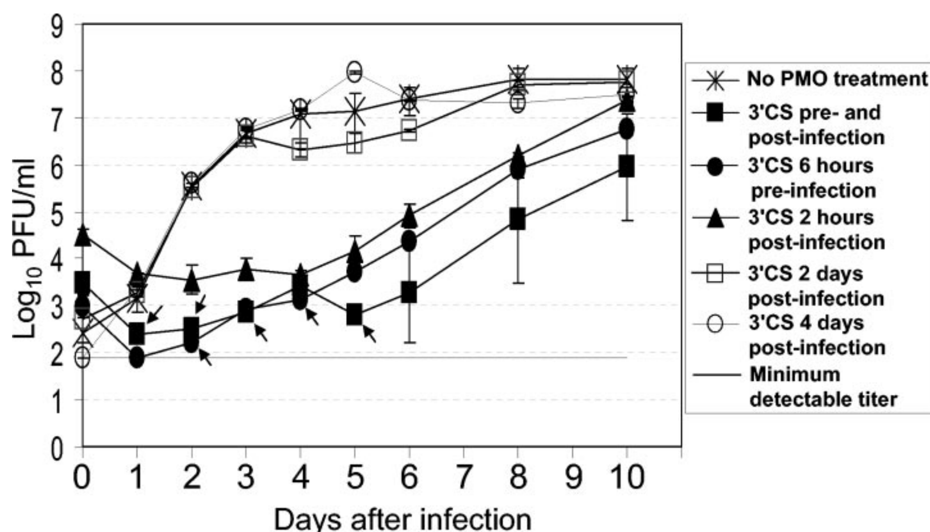

FIG. 6. Effect of time of addition of and duration of treatment with a 10 μM concentration of the P4-PMO 3′CS on DEN-2 virus replication in Vero cells. Cells were infected at an MOI of 1.0 at day 0. Data presentation, including arrows, is as described for Fig. 3. The P4-PMO regimens included (i) no P4-PMO treatment; (ii) 6-h pretreatment with 3′CS, as well as continued 3′CS treatment following viral adsorption (pre- and postinfection); (iii) only 6-h pretreatment with 3′CS, no 3′CS treatment after viral adsorption (6 h preinfection); (iv) no pretreatment with 3′CS, 3′CS added immediately after viral adsorption (2 h postinfection); (v) no 3′CS treatment until day 2 after viral infection; and (vi) no 3′CS treatment until day 4 after viral infection.

tion period, we then investigated the effect of delaying or removing 10 μM 3′CS treatment for various periods of time in relation to the time of DEN-2 virus infection. Figure 6 depicts the results of a series of six experimental regimens, each analyzing titers over a 10-day period after DEN-2 virus infection at an MOI of 1.0: (i) no P4-PMO treatment; (ii) 6-h pretreatment (6 h less than used for P4-PMO pretreatment periods in previous experiments described here) with 10 μM 3′CS P4-PMO, followed by DEN-2 virus adsorption for 2 h, three cell sheet washes, and further treatment with 10 μM 3′CS in Iscove–0% FBS medium (Fig. 6, 3′CS pre- and postinfection); (iii) only 6-h pretreatment with 10 μM P4-PMO (Fig. 6, 3′CS 6 h preinfection); and treatment with 10 μM compound starting at (iv) 2 h (Fig. 6, 3′CS 2 h postinfection), (v) 2 days (Fig. 6, 3′ CS 2 days postinfection), and (vi) 4 days (Fig. 6, 4 days postinfection) after infection. For the day 2 and 4 times of treatment, cell sheets were washed three times and then incubated with 10 μM 3′CS P4-PMO. The mean DEN-2 virus titer in non-P4-PMO-treated cells rose to approximately 7 $\log_{10}$ PFU/ml by day 3 and to approximately 8 $\log_{10}$ PFU/ml by days 8 to 10 after infection.

The results reported in Fig. 6 clearly show that early 3′CS P4-PMO treatment (before 2 days after infection) of the Vero cells was essential for suppression of DEN-2 virus replication. Pretreatment of the cells with 3′CS ensured strong suppression of virus titers in the first few days after infection. Treatment with 3′CS only immediately after viral adsorption appeared to be less successful at inhibiting viral replication during days 1 to 3 after infection, although viral titers between days 4 and 10 were only slightly higher than the viral titers following the "pre- and postinfection" and "6-h preinfection" treatments (Fig. 6). The initiation of 10 μM 3′CS P4-PMO treatment at 2 or 4 days following DEN-2 virus infection had no appreciable effect on DEN-2 virus replication relative to the DEN-2 control average viral growth curve in non-P4-PMO-treated cells.

Virus-specific immunofluorescence in Vero cells treated with P4-PMO compounds. DEN virus titers in the presence of 5 to 20 μM 3′CS or 5′SL P4-PMO were maximally inhibited to levels below the detection threshold of 80 to 160 PFU/ml in our growth curve analyses. Viral nucleic acid endured in these cultures, as indicated by viral titer rebound by day 10 in many of the treated cultures. In order to more thoroughly characterize the antiviral effect of 5′SL and 3′CS, P4-PMO-treated, virus-infected cell cultures were tested for expression of translated viral proteins. Expression of DEN-2 and WN virus-specific antigens was visualized by IFA of virus-infected Vero cells pretreated for 12 h with 5 or 7.5 μM P4-PMO compound prior to infection with DEN-2 or WN virus at an MOI of 1.0 or 0.01, respectively. Pretreated cultures were incubated again in the presence of the same P4-PMO concentration following adsorption of virus. Intense, uniform virus-specific immunofluorescence was observed in non-P4-PMO-treated Vero cell cultures at 4 days after infection with DEN-2 or WN virus (Fig. 7F and H, respectively). In the presence of 5 or 7.5 μM 3′CS P4-PMO, DEN-2 virus antigen was nearly undetectable by IFA at 4 days after infection (the result with 5 μM is shown in Fig. 7B), which correlated with the extreme suppression of DEN-2 virus replication in the presence of 5 or 10 μM 3′CS compound (Fig. 3B). On the other hand, neither the 5 μM nor the 7.5 μM concentration of the 3′CS compound exhibited any obvious effect on the intense WN virus-specific immunofluorescence detected in WN virus-infected cultures (the result with 5 μM is shown in Fig. 7G). In cultures treated with the 5 μM concentration of the 5′SL P4-PMO, DEN-2 virus antigen was detected in a few cells in several microscopic fields (one such field is shown in Fig. 7D), while treatment with the 7.5 μM concen-

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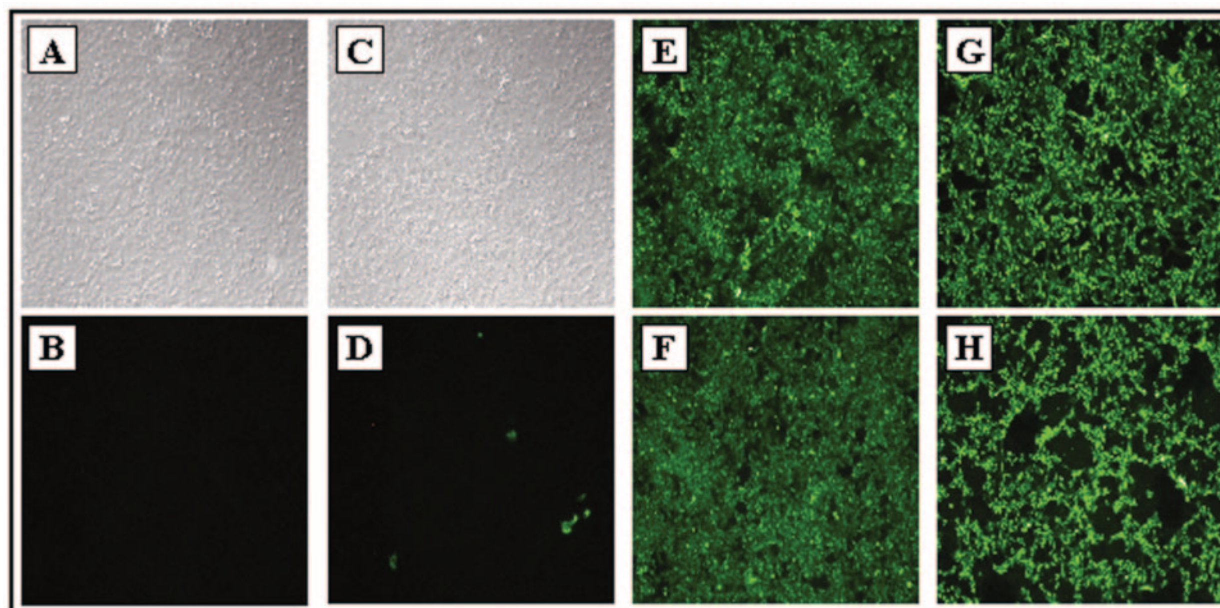

FIG. 7. Effect of P4-PMOs on DEN-2 and WN virus-specific immunofluorescence in Vero cells. (A to E and G) Cells were pretreated with 5 μM P4-PMO compound for 12 h prior to infection with virus at an MOI of 1.0 (DEN-2 virus) or 0.01 (WN virus) and incubated again with the same P4-PMO concentration after viral infection. Bright-field (A and C) and fluorescein isothiocyanate fluorescent (B and D to H) images were captured at 4 days after infection with virus. (A and B) Bright-field and fluorescent images, respectively, of the identical cell field treated with DEN-2 virus and 3′CS P4-PMO; (C and D) bright-field and fluorescent images, respectively, of the identical cell field treated with DEN-2 virus and 5′SL P4-PMO; (E) DEN-2 virus and random-sequence DScr P4-PMO; (F) DEN-2 virus control without P4-PMO treatment; (G) WN virus and 3′CS P4-PMO; (H) WN virus control without P4-PMO treatment. Viral antigen was detected with primary anti-DEN-2 New Guinea C virus mouse immune ascetic fluid (B, D, E, and F) or anti-WN Eg101 virus mouse hyperimmune ascitic fluid (G and H). Images were taken at a magnification of ×100.

tration of 5′SL essentially abolished DEN-2 antigen expression at 4 days after infection (not shown). The low number of 5′SL-treated cells expressing DEN-2 virus-specific antigen at day 4 after infection (Fig. 7D) correlated with the low viral titers but indicated some rebound in viral replication at 3 to 4 days in those cultures (Fig. 3A). DEN-2 virus-infected cultures treated with 5 or 7.5 μM random-sequence DScr P4-PMO exhibited intense, uniform DEN-2 virus-specific immunofluorescence throughout the cell culture (the result with 5 μM is shown in Fig. 7E). The 5 and 7.5 μM concentrations of the DScr compound also failed to inhibit the expression of WN virus-specific antigen whatsoever in WN virus-infected cultures (IFA results not shown). By 4 days after infection, significant CPE was evident in non-P4-PMO-treated Vero cell monolayers infected with the cytopathic WN virus (Fig. 7H). The degree of CPE induced by WN virus was much more pronounced than that which occurred as a result of P4-PMO treatment alone. WN virus-induced CPE was less pronounced in 5 or 7.5 μM P4-PMO-treated cells at 4 days after infection, as represented by the 5 μM 3′CS-treated culture in Fig. 7G. DEN-2 virus, even at a high MOI, typically exhibits little cytopathology in Vero cell cultures until late in infection. In a separate experiment, intense WN virus-specific immunofluorescence was also observed in Vero cells treated (12 h before and immediately after viral adsorption) with 10 μM 5′SL, 3′CS, or DScr P4-PMO at 2 days after infection with WN virus (data not shown). The images in Fig. 7 were captured at low magnification to permit visualization of large numbers of attached cells.

DISCUSSION

Despite an increase in the understanding of DEN virus molecular biology, immunology, and pathology, little progress has been made in the area of therapeutics for DF-DHF. Currently, no effective antiviral treatment is available for the prevention or treatment of infections with DEN virus or any other flavivirus (20). The requirements that an anti-DEN therapeutic be nontoxic, inexpensive, easy to administer, stable for months at variable temperatures, and effective against all four DEN virus serotypes present an immense challenge to drug developers. PMOs and peptide-PMOs have inherent properties that may satisfy most of these requirements (13, 17, 28).

Identification of highly active P4-PMO compounds. The purpose of this investigation was to test peptide-PMO antisense compounds targeted to regions of the DEN virus genome that have been established in the literature as critical to the flavivirus life cycle. PMOs alone do not readily cross the plasma membranes of cultured cells, and therefore some type of assisted delivery is required in order to use them effectively in cell culture experiments (28). PMOs conjugated to an arginine-rich peptide, such as the $R_5F_2R_4$-PMO (P4-PMO) compound used in this study, have the appealing characteristic of achieving uptake into mammalian cells in culture by simple incubation for a few hours at 37°C and achieving subsequent distribution and functional ability in both nuclear and cytoplasmic compartments (28, 30). Six P4-PMO compounds were synthesized and compared for efficacy and specificity against

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DEN-2 virus as assessed by plaque assays. An initial survey of all six compounds at 20 μM showed that the random-sequence P4-PMO DScr and DEN-2 virus-specific antisense P4-PMOs AUGp (targeting the AUG initiation codon for translation of the viral polyprotein), 5′CS (5′ cyclization sequence), and 3′SL (3′-terminal nucleotides of the viral genome) reduced the viral titer in DEN-2 virus-infected cells by 0.1 to 1.8 $\log_{10}$ PFU/ml at 6 days after viral infection. Two other P4-PMO compounds, 5′SL and 3′CS, targeting the 20 5′-terminal bases and 3′ cyclization sequence region, respectively, suppressed DEN-2 virus titers by more than 5.7 $\log_{10}$ PFU/ml, below the detection limit of 160 PFU/ml for this experiment. Each of the six P4-PMO compounds induced some degree of CPE in uninfected cells at a concentration of 20 μM. Nevertheless, highly robust replication of DEN-2 virus to titers greater than 6.0 $\log_{10}$ occurred in the presence of the 20 μM AUGp, 5′CS, 3′SL, and control DScr compounds. Based on their apparent exquisite antiviral effects, we further investigated the antisense activities of the 5′SL and 3′CS P4-PMO compounds.

Specificity of active anti-DEN virus compounds. In this study, P4-PMO treatment induced dose-dependent CPE in uninfected Vero cells. Such cytotoxicity could have concomitant deleterious effects on viral replication. The random-sequence DScr P4-PMO, which exhibited a CPE that was at least as pronounced as those shown by the 5′SL and 3′CS compounds in uninfected cells, was included in each experiment to control for such non-virus-specific cellular and antiviral effects. Non-virus-specific cellular CPE, as well as nonspecific antiviral effects, was less evident at P4-PMO concentrations of below 20 μM, particularly at 10 μM or lower as assessed by the minor effect of the random-sequence DScr P4-PMO on DEN-2 virus titers (Fig. 3), by visual CPE scoring of uninfected cell sheets (data not shown), by MTT cell viability assays (Fig. 4), and by the minimal effect of 10 μM 3′CS compound on viral replication when cells were first treated with this P4-PMO at 2 or 4 days after infection with DEN-2 virus (Fig. 6). The DEN virus-targeted P4-PMOs acted similarly to the DScr control P4-PMO. Neither the 5′SL nor the 3′CS compound had any discernible antiviral effect at 1 μM. Therefore, the majority of experiments in this study were performed at 5 to 10 μM P4-PMOs 5′SL, 3′CS, and DScr. DEN-2 virus titers were decreased by 1.0 $\log_{10}$ PFU/ml or less in the presence of 10 μM DScr, while DEN-1, DEN-3, and DEN-4 virus titers were suppressed by 0.7 to 2.2 $\log_{10}$ PFU/ml, relative to non-P4-PMO-treated, DEN virus-infected cultures. The apparent greater suppressive activity of DScr in DEN-1, -3, and -4 virus-infected cells was likely due to additive effects of the DScr compound and the more robust early replication of these three DEN virus serotypes.

Both 5′SL and 3′CS P4-PMOs generated clear dose-dependent, virus-specific antiviral effects in DEN virus-infected Vero cells. In growth curves obtained with cells infected at an MOI of 1.0, 10 μM 5′SL and 3′CS inhibited replication of DEN-1 to -4 viruses, whereas the heterologous flavivirus WN virus NY99 exhibited the highly robust replication observed for this virus in non-P4-PMO-treated cells. Even at the low MOI of 0.01, WN virus replicated efficiently in the presence of 5 or 7.5 μM 5′SL and 3′CS, as shown by IFA (Fig. 7). At 10 μM, the 5′SL compound affected replication of DEN viruses and exhibited an order of effectiveness of inhibition of DEN-2 virus > DEN-1 virus > DEN-3 virus > DEN-4 virus (Fig. 2 and 3). 5′SL-specific inhibition of DEN-4 virus replication was more effective by only about 1 $\log_{10}$ reduction in viral titer than the random-sequence DScr compound. The decreased effectiveness of 5′SL against DEN-4 virus was likely due to the presence of the contiguous two-nucleotide mismatch between the antisense sequence of 5′SL and the target 5′-terminal sequence in DEN-4 virus (Table 1). The 5′SL and 3′CS P4-PMO antisense sequences were specific for their targeted sense sequences in the DEN-2 virus genome. These two P4-PMO sequences did not match any other DEN-2 genome or antigenome sequence at greater than 70% identity. The DScr sequence was likewise unique.

It is noteworthy that the 3′ cyclization domain of WN virus is very similar in sequence to the conserved 3′ cyclization domains of DEN-1 to -4 viruses (Table 1). However, WN virus has an additional base and one contiguous mismatch compared to the same region in DEN-1 to -4 viruses. This single-base "insertion" has the effect of offsetting the 3′ portion of the WN virus target region by one nucleotide in relation to the DEN virus-specific 3′CS P4-PMO. Although the 5′SL P4-PMO exhibited detectable activity against DEN-4 virus (Fig. 5C), with whose target sequence it has two internal contiguous mismatches, the 3′CS P4-PMO lacked significant activity against WN virus. The 3′CS compound is an 18-mer and has an alignment with WN virus that consists of 12 contiguous bases that are offset by one base in relation to WN virus. In this case, an additional single RNA base and an additional single-nucleotide mismatch in an otherwise complementary sequence between the target RNA and P4-PMO was sufficient to greatly reduce P4-PMO efficacy. After rapid achievement of peak viral titers in Vero cells, the decay of WN virus titers was delayed in the presence of the 5′SL, 3′CS, and DScr compounds (Fig. 5D). This modulation of WN virus replication appeared to be due to a non-virus-specific effect of the 10 μM P4-PMO treatment. Except for the 3′CS-targeted domain just described, none of these three P4-PMO compounds had greater than 72% sequence identity with the WN virus genome or antigenome.

The data in Fig. 5 show that a single treatment with the 5′SL or 3′CS P4-PMO in the 5 to 10 μM range had an anti-DEN-2 virus effect of over 99% for several days. Concomitantly, 10 μM concentrations of these compounds resulted in a 10 to 20% loss of cell viability (Fig. 4). This level of antiviral efficacy in relation to nonspecific cytotoxicity indicates a specificity index of at least one order of magnitude. The predictive value of this assessment from cell culture experiments to pharmacological behavior in vivo is limited, however.

The 3′CS P4-PMO is the most active anti-DEN virus compound. Of the two highly effective P4-PMOs, 3′CS was clearly the more potent anti-DEN virus compound. This compound reduced the titers of all four serotype (DEN-1 to -4 viruses) to below the growth curve assay detection limit of 80 PFU/ml for 4 days or longer after viral infection. Although the 3′CS compound was highly effective against all four serotypes of DEN virus, a degree of DEN virus serotype specificity was observed, even though the 3′CS-targeted viral genomic sequences were identical in all four DEN virus serotypes. At 10 μM 3′CS P4-PMO, DEN-2 virus titers were suppressed to below the detectable limit for at least 10 days, followed by DEN-1 (8

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days), DEN-3 (4 to 6 days), and DEN-4 (3 to 4 days) viruses, in P4-PMO-treated Vero cultures infected at an MOI of 1.0 with each DEN virus serotype. This degree of heterogeneity in the level of viral inhibition may indicate modulation of the P4-PMO effect by the DEN virus serotype-specific sequence context or structure surrounding the domain targeted by the 3′CS P4-PMO.

Differential activity of similar compounds. Two of the P4-PMO compounds used in this study targeted the 5′ end of the DEN-2 virus genome. 5′SL was directed to the 20 5′-proximal bases of the DEN-2 virus 5′UTR and was highly active against three of four serotypes, whereas the AUGp compound, directed to the translation start site region, was relatively ineffective in its only trial, against DEN-2 virus. This was an unexpected result given the reported reliability of the translation initiation codon region as a PMO target site in general (29, 40). Both the 5′SL and AUGp target sites are located within putative stem-loops with separate but similar structures. The 5′SL target lies within a stem-loop structure of 69 bases that is structurally conserved across all members of the *Flavivirus* genus (1, 37, 42) and that includes strong sequence conservation across DEN virus genotypes (19). This part of the genome is thus likely to play a critical role in the viral life cycle, probably in genome translation. Its function may involve the recruitment of cellular and viral proteins that bind the 5′ end of DEN-4 virus (5). Cahour et al. (3) found that the deletion of bases 18 to 43 lowered the translation efficiency and replication of DEN-4 virus RNA. We interpret the high efficacy of 5′SL as evidence of the ability of this compound to invade and disrupt the 5′-proximal stem-loop, as well as of the importance of the 5′-proximal stem-loop region for efficient DEN amplification. The reasons for the ineffectiveness of the AUGp P4-PMO are unknown but may be due to the inaccessibility of its RNA target site. Oddly, the stem-loop region that contains the AUG start site is predicted to be less stable than the stem-loop at the 5′ terminus of the same strand that contains the target of 5′SL (1). Since there is little clear evidence in the literature of RNA tertiary structure at either site, we speculate that the limited efficacy of AUGp may be due to hindrance at that target site by some viral and/or cellular factor. It is unlikely that AUGp was being decoyed by a host cell RNA sequence of high complementarity, since a BLAST homology search returned no primate sequence that would be a candidate for such an interaction.

Two of the P4-PMOs studied targeted the viral cyclization sequences, but only one of these (3′CS and not 5′CS) was effective in suppressing DEN-2 virus titers. Flavivirus cyclization sequences have known roles in supporting RNA replication (22, 44), and there is no obvious basis for differential efficacy between 5′CS and 3′CS in interfering with replication. The 5′CS and 3′CS compounds had similar lengths, GC contents, and design rationales. Several influences may have contributed to the large difference in anti-DEN virus activity observed between these two compounds. One possibility is that there exists a conformational inaccessibility of the DEN virus RNA target sequence to 5′CS, in a fashion similar to that suggested for AUGp above. Another explanation is invited by the genomic locations of the target sequences of these two compounds in relation to the nature of the PMO mechanism of action. It has been reported that PMOs targeted to RNA sequence that resides more than about 20 bases downstream from the translation start sites of eukaryotic genes are rarely effective at inhibiting translation (38, 40). This lack of efficacy is presumably due to the processive action of translocating ribosomes in dislocating bound PMO from target RNA. 5′CS targets an RNA sequence in the DEN virus ORF located 30 to 50 bases downstream from the translation initiation codon. We assume that 5′CS bound to this site would be forcibly dislodged from viral RNA during the translation process, whereas 3′CS, which targets sequence located >300 bases downstream of the UAG translation termination codon, would not be. It is perhaps incongruous to suggest that a translation-associated event, that of the processing ribosomal complex, can explain the difference between the activities of two compounds that are both designed to work by interfering with genome cyclization, an event that occurs during the viral replication process. The more frequent removal of 5′CS than of 3′CS from viral RNAs by the translational machinery, however, may have consequence for their respective anti-DEN virus efficacies. It is also possible that the duplex of 3′CS and its target RNA may be disruptive to a viral molecular event other than the cyclization process. While future experiments are needed to identify the inhibitory mode of action of the 3′CS (and 5′SL) P4-PMO, it remains a novel observation that a PMO compound targeted to a sequence located in a viral 3′ UTR shows such profound efficacy at inhibiting viral amplification.

DEN virus titer eventually rebounds after treatment. The effective suppression of DEN-2 virus titer following 3′CS pretreatment alone, without continued P4-PMO treatment following viral adsorption, indicates an intracellular antiviral effect of the compound (6-h preinfection regimen in Fig. 6). DEN-2 virus replication rebounded after 6 days in cultures in which early viral replication was blocked for 4 to 6 days by 5 μM 3′CS P4-PMO treatment (Fig. 3B and 7B). Such a rebound in viral titer indicates that intact viral genomes persisted in at least a portion of the P4-PMO-treated, virus-infected cells until intracellular conditions permitted translation of the viral polyprotein and replication of the viral genome. It is possible that the P4-PMO compounds were intracellularly sequestered, degraded, or effluxed after prolonged incubation in Vero cells. An arginine-rich peptide would likely undergo rapid degradation once inside a cell, and effluxed P4-PMO alone would not be expected to reenter cells. Another possible explanation for at least some instances of viral rebound is the evolution of escape variants which contain mutations in the sequence targeted by the P4-PMO. The severe suppression of DEN virus replication evidenced in our results represents a strong selective mechanism for the development of such variants. We did not investigate the possibility of escape variants in this study. We also did not investigate the possibility that prolonging the exposure of infected cells to a P4-PMO compound by multiple treatments, through periodic replacement of cell culture medium containing an effective antiviral concentration of 5′SL or 3′CS, might completely cure the cell culture of DEN virus infection.

Early treatment is required for efficacy. Investigation of the effect of the timing of 10 μM 3′CS administration to Vero cells in relation to the time of DEN-2 virus inoculation showed clearly that effective suppression of viral amplification required P4-PMO to be present before or soon after infection (Fig. 6).

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A comparison in which 3′CS treatment commenced at four sequential points in time in relation to the period of DEN-2 virus adsorption revealed that the earlier that the 3′CS P4-PMO was administered to cells in relation to virus infection, the greater was its antiviral effect. 3′CS administration beginning at either 2 or 4 days after infection had little or no anti-DEN virus effect. The requirement that 3′CS be present early in the DEN virus infection process of a given cell in order to be effective may have implications for its ultimate clinical utility.

It is apparent from this study that there exist at least two locations in the genomic RNAs of DEN viruses that represent productive targets for PMO compounds. Targeting of P4-PMO to the DEN virus 5′-proximal nucleotides or 3′ cyclization sequence resulted in a profound reduction in viral replication. The magnitude and duration of suppression of the in vitro replication of all four DEN virus serotypes shown by the 3′CS and 5′SL P4-PMOs clearly suggest that these particular compounds be considered for further evaluation, including in vivo study. Furthermore, investigation of the impact of P4-PMOs targeted to specific viral RNA regions could have considerable value in functional analyses of RNA sequences and structures.

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