

## Distinct RNA Elements Confer Specificity to Flavivirus RNA Cap Methylation Events<sup>∇</sup>

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**The 5' end of the flavivirus plus-sense RNA genome contains a type 1 cap (m<sup>7</sup>GpppAmG), followed by a conserved stem-loop structure. We report that nonstructural protein 5 (NS5) from four serocomplexes of flaviviruses specifically methylates the cap through recognition of the 5' terminus of viral RNA. Distinct RNA elements are required for the methylations at guanine N-7 on the cap and ribose 2'-OH on the first transcribed nucleotide. In a West Nile virus (WNV) model, N-7 cap methylation requires specific nucleotides at the second and third positions and a 5' stem-loop structure; in contrast, 2'-OH ribose methylation requires specific nucleotides at the first and second positions, with a minimum 5' viral RNA of 20 nucleotides. The cap analogues GpppA and m<sup>7</sup>GpppA are not active substrates for WNV methyltransferase. Footprinting experiments using Gppp- and m<sup>7</sup>Gppp-terminated RNAs suggest that the 5' termini of RNA substrates interact with NS5 during the sequential methylation reactions. Cap methylations could be inhibited by an antisense oligomer targeting the first 20 nucleotides of WNV genome. The viral RNA-specific cap methylation suggests methyltransferase as a novel target for flavivirus drug discovery.**

The 5' cap is essential for mRNA stability and efficient translation initiation (14, 22). The cap of cellular mRNAs is formed in the nucleus through three enzymatic modifications. The 5' triphosphate of nascent pre-mRNA is hydrolyzed to a 5' diphosphate by an RNA triphosphatase, the diphosphate RNA end is capped with GMP by an RNA guanylyltransferase, and the GpppN cap is methylated at the N-7 position of guanine by an RNA guanine-methyltransferase (N-7 MTase), resulting in type 0 cap (m<sup>7</sup>GpppN) (27). The first and second nucleotides of many cellular and viral mRNAs are further methylated at the ribose 2'-OH position by a nucleoside 2'-O MTase, to form type 1 cap (m<sup>7</sup>GpppNm) and type 2 cap (m<sup>7</sup>GpppNmNm) structures, respectively (15). Cap methylations use *S*-adenosyl-L-methionine (SAM) as a methyl donor and generate *S*-adenosyl-L-homocysteine (SAH).

Viruses represent an attractive system for studying RNA capping. The capping mechanisms of some viral mRNAs are different from those of the cellular mRNA (15). For Semliki Forest virus (a plus-strand RNA virus within the alphavirus-like superfamily), GTP is first methylated to m<sup>7</sup>GTP; the m<sup>7</sup>GMP moiety of m<sup>7</sup>GTP is then transferred to the 5' diphosphate end of the viral RNA to form a type 0 cap structure (2). For vesicular stomatitis virus (a minus-strand RNA virus from the family *Rhabdoviridae* within the order *Mononegavirales*), the type 1 cap of viral mRNA is formed through transfer of a GDP moiety from GTP to the 5' monophosphate of the acceptor RNA (1), with subsequent methylations at the 2'-O and guanine N-7 positions of the cap (21, 29). The differences

between host and viral cap formation could potentially be used for development of antiviral therapy.

Many members of the genus *Flavivirus* are significant human pathogens, including dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus, and the tick-borne encephalitis virus (18). Flaviviruses contain a single-strand, plus-sense RNA genome approximately 11 kb in length. A single open reading frame encodes a polyprotein that is processed by viral and cellular proteases into three structural proteins and seven nonstructural proteins (18). The 5' and 3' untranslated regions (UTRs) of the flavivirus genome are approximately 100 nucleotides and 400 to 700 nucleotides in length, respectively. Besides several conserved sequence elements, both the 5' and 3' terminal sequences of the genomic RNA form highly conserved stem-loop structures (5, 6). The conserved sequence elements and stem-loop structures may function to modulate the viral life cycle through interactions with viral-host proteins and through RNA-RNA interactions. However, the mechanisms of these modulations remain unknown.

Like many cytoplasm-replicating viruses, flaviviruses encode some, if not all, of their own capping machinery. Although the identity of guanylyltransferase remains elusive, the flavivirus NS3 and NS5 encode RNA triphosphatase (17, 30) and 2'-O ribose MTase activities (12), respectively. We recently showed that WNV NS5 could methylate both the N-7 and 2'-O positions of the viral RNA cap and that the two methylation events are sequential, with N-7 methylation preceding 2'-O methylation (25). Remarkably, cap methylation by WNV NS5 was specific for viral RNA sequence; neither MTase activity was detected on GpppA- or m<sup>7</sup>GpppA-terminated plasmid-derived RNA (25). This is in contrast to cellular cap MTases, whose methylation activity is not RNA sequence specific (19).

Here we report that NS5 MTases from four serocomplexes

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TABLE 1. Primer sequences<sup>a</sup>

Primer	Sequence (5'-3')
1.....	TAATATCATATGGGCACGGAGCCCAAGGGGAAACACTG
2 <sup>b</sup> .....	CATCATCTCGAGTCTTGTTCAGCGCCTAAGTCCACGCTCT CTTTCGTATGTTGGCTTC
3.....	TAATATCATATGGGCACGGAGCCCAAGGGGAAACACTG
4.....	TATATTGTCGACGCGTGTCCCAATTGGGAGGATGACGCTC
5.....	TATATTCCATATGGGTGGAGCAGAGGGGAAGCACC
6.....	TATATTGTCGACCGTGTTCAGTTCAGTTCAGGTCGATC
7 <sup>c</sup> .....	CAGTAATACGACTACTATTAGTAGTTCCGCTGTGTGAGCT GACAAACTT
8.....	TCTTCAGTCCAATCAAGGACAACACGC
9.....	TGTCCTACGAGTTGCATG
10.....	AGCTCGCACTGTGTTAAT
11.....	CTACTAAGTTTGTGACGCTC
12.....	TGTCAGCTCACACAGG
13.....	GCTCACACAGGCGAAGCTACT
14 <sup>d</sup> .....	CACAGGCGAACTACTAATAG
15 <sup>e</sup> .....	CAGTAATACGACTACTATTAGTAGTTTCTTGCACGTTGTG CGGGTGC
16.....	CTGGTTCGCGACTTATTTGCGGTCACTTTAAG
17 <sup>e</sup> .....	CGTACGACAGTAATACGACTACTATTACATTAATTGCGTT GCGCTCAC
18.....	CCGCTTTGAGTGAGCTGATACCGC

<sup>a</sup> Annotations and usage of the primers are described in Materials and Methods.

<sup>b</sup> The underlined sequence represents a silent mutation to knock out an internal NdeI site near the C terminus of the DENV-1 MTase-coding sequence.

<sup>c</sup> The italicized sequence represents a bacteriophage T7 class II  $\phi$ 2.5 promoter that was used to synthesize the ATP-initiated RNA (underlined). Three extra nucleotides ("CAG") were added to the 5' end of the promoter sequence.

<sup>d</sup> The 3'-terminal four nucleotides (italicized) are complementary to the most 3' sequence of the T7 class II  $\phi$ 2.5 promoter.

of flaviviruses (DEN, YF, JE, and TBE) specifically methylate the cap structure of flaviviral RNA. Using WNV as a model, we have defined distinct elements within the 5' stem-loop of viral RNA that are required for each step of the N-7 cap and 2'-O ribose methylations. The N-7 cap methylation requires a 5' stem-loop structure and guanine and uracil at positions 2 and 3, respectively; in contrast, 2'-O ribose methylation requires a minimum viral RNA length of 20 nucleotides and adenine and guanine at positions 1 and 2, respectively. Footprinting experiments showed that NS5 protects the 5'-terminal nucleotides of viral RNA during the two methylation reactions. Furthermore, an antisense oligonucleotide complementary to the 5'-terminal 20 nucleotides of the WNV genome inhibits both the N-7 cap and 2'-O ribose methylations.

## MATERIALS AND METHODS

**Cloning, expression, and purification of flavivirus MTases.** Recombinant MTases from Powassan virus (PWV), WNV, DENV-1, and YFV contained the N-terminal 264, 300, 262, and 266 amino acids of NS5 protein, respectively. Each protein had a His tag to facilitate purification: WNV MTase contained an N-terminal His tag, whereas PWV, DENV-1, and YFV MTases had a C-terminal His tag. WNV MTase was prepared as described previously (25). For cloning of DENV-1 MTase, a DNA fragment representing the MTase domain was PCR amplified from a DENV-1 replicon cDNA (24) using primers 1 and 2 (Table 1). Primers 1 (forward) and 2 (reverse) contained a 5' tail with NdeI and XhoI recognition sites, respectively. Primer 2 also had a silent mutation (A→G change, underlined) to knock out an internal NdeI site (nucleotide positions 8322 to 8327; GenBank accession no. U88535) near the C terminus of the DENV-1 MTase-coding sequence. The DENV-1 PCR product was then cloned into expression plasmid pET26b(+) (Novagen) at NdeI and XhoI sites. For cloning of YFV MTase, primers 3 and 4 (Table 1) were used to PCR amplify the MTase-coding sequence from a YFV infectious cDNA clone (4); similarly, the resulting PCR product was cloned into pET26b(+) at the NdeI and XhoI sites. For PWV, the MTase-coding region was reverse transcription-PCR amplified from genomic RNA using primers 5 and 6 (Table 1). Since the coding sequence of the PWV

MTase has an internal XhoI site (GenBank accession no. NC\_003687), primer 6 (reverse) contained a 5' SalI site (produces compatible overhang with XhoI site). The PWV MTase PCR product was also cloned into pET26b(+) at the NdeI and XhoI sites. All plasmid inserts were sequenced to ensure that no mutations had occurred during cloning. The DENV-1, YFV, and PWV MTases were expressed in *Escherichia coli* strain Rosetta 2(DE3)/pLysS (Novagen) and purified through a nickel-nitrioltriacetic acid column, followed by a gel filtration 16/60 Superdex column (Amersham), as previously reported for WNV MTase (25). The preparation of full-length NS5 of WNV (see Fig. 6A) was described previously (31).

**Preparation of RNA substrates.** WNV RNA representing the first 190 nucleotides of the genome (see Fig. 1) was in vitro transcribed from PCR products by using an infectious cDNA clone as a template (26) and primers 7 and 8 (Table 1). Since flavivirus genomes start with an adenine, a bacteriophage T7 class II  $\phi$ 2.5 promoter (italicized in Table 1) was used to facilitate synthesis of ATP-initiated RNA (the underlined A in primer 7, Table 1) using T7 RNA polymerase (10). Mutant RNAs containing single nucleotide substitutions within the first four residues of WNV genome (see Fig. 3) were similarly prepared using a modified primer 7 (containing the substituted nucleotides) and primer 8 for PCR. RNAs representing the first 74, 35, 25, 20, and 15 nucleotides of WNV genome (see Fig. 4) were transcribed from PCR products amplified using forward primer 9 and respective reverse primers 10, 11, 12, 13, or 14. Primer 9 is located in the plasmid vector (pBR322) at a position 210 nucleotides upstream of the T7 promoter. For mutagenesis of the 5' stem-loop of WNV RNA (see Fig. 5), a cDNA fragment spanning the first 190 nucleotides of the genome was cloned into vector pGEM-9Zf(-) (Promega) at the BamHI and NsiI sites, resulting in plasmid pWNV190nt. A QuikChange II XL site-directed mutagenesis kit (Stratagene) was used to engineer indicated mutations in pWNV190nt. The mutant plasmids were verified by DNA sequencing and served as templates for PCR with primers 7 and 8. The PCR products were then transcribed into RNA by using a MEGAshortscript kit (Ambion).

For preparation of PWV RNA (Fig. 1C), a DNA fragment representing the first 190 nucleotides of the genome was reverse transcription-PCR amplified from genomic RNA and TA cloned into vector pCRII-TOPO (Invitrogen), resulting in plasmid pPWV190nt. The pPWV190nt DNA was then used as a template for PCR amplification using forward primer 15 (containing a T7 promoter, italicized) and reverse primer 16 (Table 1). For the preparation of plasmid RNA (Fig. 1D), forward primer 17 (with a T7 promoter, italicized) and reverse primer 18 (Table 1) were used to amplify DNA spanning nucleotides A574 to G763 of pUC19 (GenBank accession no. X02514). For the preparation of WNV and pUC chimeric RNAs (see Fig. 2), PCR was performed by using a forward primer (containing a T7 promoter followed by the designed chimeric sequences) and an appropriate reverse primer (primer 8 or 18 for RNA with a WNV or pUC sequence, respectively). All PCR products were extracted with phenol-chloroform, precipitated with ethanol, and transcribed into RNAs as described above.

In vitro synthesized RNAs were capped using [ $\alpha$ -<sup>32</sup>P]GTP (Amersham) and a vaccinia virus capping enzyme (Ambion) in the presence or absence of cold SAM according to the manufacturer's instructions. The resulting 5'-labeled G\*pppA- and m<sup>7</sup>G\*pppA-RNA (the asterisk indicates that the following phosphate is <sup>32</sup>P labeled) were used as substrates for N-7 and 2'-O methylations, respectively. For the removal of unincorporated [ $\alpha$ -<sup>32</sup>P]GTP, the capping reactions were passed through two G-25 size columns (Ambion), extracted with phenol-chloroform, and precipitated with ethanol. RNA secondary structures were calculated through minimization of free energy using the MFold program in the GCG package (Genetics Computer Group).

**Cap methylation assays.** The N-7 methylation was performed in a 20- $\mu$ l reaction (containing 50 mM Tris [pH 7.0], 50 mM NaCl, 2 mM dithiothreitol [DTT], 10<sup>6</sup> cpm of G\*pppA-RNA, 80  $\mu$ M SAM, and 1  $\mu$ g of MTase) at 22°C for 5 min. 2'-O methylation was performed in a 20- $\mu$ l reaction (50 mM glycine [pH 10], 2 mM DTT, 10<sup>6</sup> cpm of m<sup>7</sup>G\*pppA-RNA, 80  $\mu$ M SAM, and 1  $\mu$ g of MTase) at 22°C for 1 h. Since the N-7 buffer efficiently supports N-7, but not 2'-O methylation, reactions in the N-7 buffer allowed us to stage the N-7 methylation without progressing into the 2'-O methylation (33). Control m<sup>7</sup>G\*pppGm-RNA (Fig. 3B, left panel) was prepared by incubating the vaccinia virus VP39 protein in a 20- $\mu$ l reaction containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 50  $\mu$ M SAM, 5  $\times$  10<sup>5</sup> cpm of m<sup>7</sup>G\*pppG-RNA, and 0.5  $\mu$ g of recombinant VP39 for 1 h at 30°C. All methylation reactions were digested with nuclease P1, analyzed on polyethyleneimine cellulose thin-layer chromatography (TLC) plates (JT Baker) using a solvent of 0.65 M LiCl or 0.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and quantified by autoradiography or with a PhosphorImager (25). Results representative of two to three independent experiments were presented.

**RNA footprinting.** G\*pppA-RNA and m<sup>7</sup>G\*pppA-RNA substrates representing the 5'-terminal 190 nucleotides of WNV genome were used (described

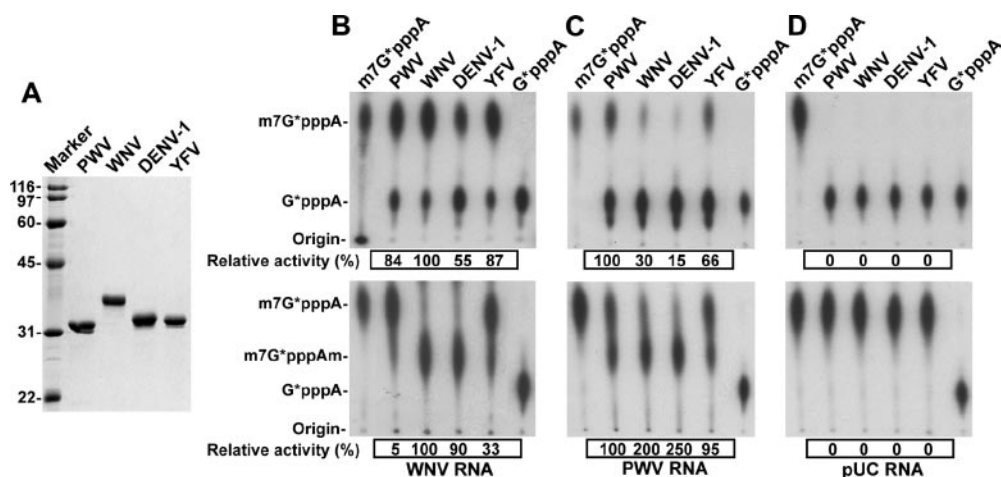


FIG. 1. Specific cap methylation by flavivirus MTase. (A) Recombinant MTases of PWV, WNV, DENV-1, and YFV contained a His tag and the N-terminal 264, 300, 262, and 266 amino acids of NS5, respectively. The purified proteins were analyzed by SDS-PAGE and stained with Coomassie blue. Each MTase was tested for its N-7 and 2'-O methylation activities in three RNAs containing sequences from WNV (B), PWV (C), and a plasmid (D). WNV and PWV RNAs contain the 5'-terminal 190 nucleotides of the viral genome; the 190-nucleotide plasmid RNA is derived from pUC19 (positions A574-G763; GenBank accession no. X02514). For N-7 methylation (top portions of panels B to D), substrate G\*pppA-RNA was incubated with the indicated MTases in the N-7 buffer that is optimal for N-7 methylation but not for 2'-O methylation (33). For 2'-O methylation (bottom portions of panels B to D), m<sup>7</sup>G\*pppA-RNA was incubated with the indicated MTase. All reactions were then digested by nuclease P1 (to release G\*pppA, m<sup>7</sup>G\*pppA, and m<sup>7</sup>G\*pppAm), analyzed on TLC plates, and visualized by autoradiography. The positions of the origin, and the migrations of the G\*pppA, m<sup>7</sup>G\*pppA, and m<sup>7</sup>G\*pppAm molecules are indicated on the left. The mobility of m<sup>7</sup>G\*pppAm on the TLC plates was previously determined by VP39-mediated 2'-O methylation of m<sup>7</sup>G\*pppA-RNA (25); the mobility of G\*pppA and m<sup>7</sup>G\*pppA was validated with cold markers under UV shadowing. See Materials and Methods for experimental details.

above) to probe the RNA-NS5 interactions prior to the N-7 and 2'-O methylations, respectively. Footprinting reactions (a total volume of 10  $\mu$ l) contained 10<sup>6</sup> CPM of G\*pppA-RNA or m<sup>7</sup>G\*pppA-RNA, 50 mM Tris (pH 7.0), 50 mM NaCl, 2 mM DTT, 4 or 10  $\mu$ g of WNV full-length NS5, and 0.5 or 1 U of RNase I (New England Biolabs). After incubation at room temperature for 3 min, the reactions were stopped with 10  $\mu$ l of denaturing loading buffer, heated at 95°C for 1 min, separated on a 20% urea-polyacrylamide gel, and analyzed by autoradiography. The hydrolysis ladder was prepared by incubation of 10<sup>6</sup> cpm of WNV RNA with 10  $\mu$ g of yeast RNA in an alkali hydrolysis buffer (Ambion) at 95°C for 10 min. The G ladder was generated by digestion of 10<sup>6</sup> cpm of WNV RNA and 10  $\mu$ g of yeast RNA with 1 U of RNase T<sub>1</sub> in an RNA sequencing buffer (Ambion) at room temperature for 8 min.

**Inhibition of methylation by an antisense oligomer.** An antisense phosphorodiamidate-morpholino-oligomer (PMO) that is complementary to the first 20 nucleotides of the WNV genome (5'-end PMO) (11) was examined for its inhibition of RNA cap methylation (see Fig. 7). The sequence and backbone of 5'-end PMO were reported previously (11). A scrambled PMO containing a random sequence was used as a negative control. To test the effects of PMOs on cap methylation, we preincubated the PMOs with standard methylation components (described above) without MTase at 60°C for 1 min, followed by cooling to room temperature for 10 min. WNV MTase (1  $\mu$ g) was then added to initiate methylation reactions. The reactions were performed as standard methylation assays, digested with nuclease P1, and analyzed on TLC plates (25).

## RESULTS

**Cap methylation by flavivirus NS5 is specific to the viral RNA.** We previously demonstrated that WNV possesses a cap-dependent MTase activity within the N-terminal 300-amino-acid segment of NS5 and that the C-terminal RNA-dependent-RNA polymerase (RdRp) domain is not required for the MTase activity (25). To determine whether this MTase activity is universal across the genus *Flavivirus*, we cloned, expressed, and purified YFV, DENV-1, WNV, and PWV MTase domains in bacteria, each as a His-tagged fusion protein (Fig. 1A). The selected viruses represent four serocomplexes of flaviviruses: YF, DEN, JE, and TBE, respectively. The recombinant PWV,

WNV, DENV-1, and YFV MTase proteins contained the N-terminal 264, 300, 262, and 266 amino acids of the NS5 protein, respectively. Note that the mobility of the MTases in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) did not directly correlate with the size of the polypeptide, probably due to differences in amino acid composition among the four proteins. Initially, we used a WNV RNA, containing the 5'-terminal 190 nucleotides of the genome, to assay for methylation activities of the four MTases. G\*pppA-RNA and m<sup>7</sup>G\*pppA-RNA were used as substrates in separate assay conditions to allow for the N-7 and 2'-O methylations, respectively (see Materials and Methods). MTases from YFV, DENV-1, or PWV could efficiently methylate WNV G\*pppA-RNA at the N-7 position, reaching 55 to 87% of the WNV MTase activity (Fig. 1B, top panel). In contrast, when YFV, DENV-1, or PWV MTase was incubated with WNV m<sup>7</sup>G\*pppA-RNA, the 2'-O ribose methylation activity was lower than that of the WNV MTase; among them, PWV MTase exhibited the lowest activity, at only 5% of that derived from the WNV MTase (Fig. 1B, bottom panel).

The low 2'-O activity of PWV MTase on WNV RNA could potentially result from two factors: (i) the PWV enzyme had an intrinsically low 2'-O methylation activity and/or (ii) the PWV MTase required a cognate viral RNA substrate to achieve an efficient methylation. To test these possibilities, we prepared a PWV RNA derived from the 5'-terminal 190 nucleotides of the genome. Compared to the PWV enzyme, the MTases from WNV, DENV-1, and YFV exhibited lower N-7 activities but still higher 2'-O activities on the PWV RNA (Fig. 1C). To further examine the specificity of cap methylation, we prepared a nonviral RNA of 190 nucleotides derived from a plasmid pUC19. Neither N-7 nor 2'-O methylation was detected when

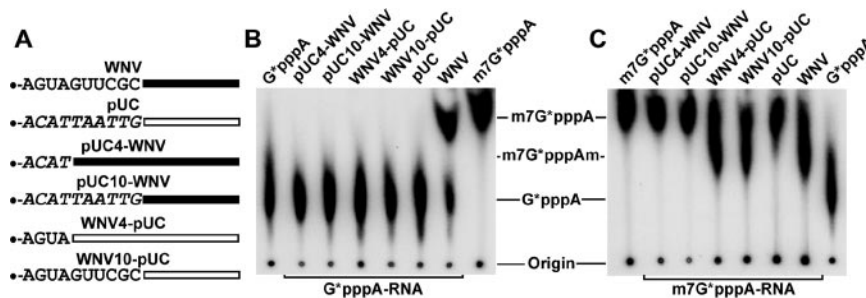


FIG. 2. Effects of 5'-terminal 4 and 10 nucleotides of WNV genome on N-7 and 2'-O methylations. (A) WNV, plasmid pUC, and four chimeric RNA substrates. All RNAs were 190 nucleotides in length. The first 4 or 10 nucleotides were swapped between the WNV RNA and the plasmid pUC RNA. The WNV and pUC RNAs are represented by filled and unfilled boxes, respectively. The 5'-terminal sequences for WNV and pUC RNAs are indicated in regular type and italics, respectively. All RNAs contained a 5' cap structure indicated by a filled circle (●). For each RNA, substrates G\*pppA-RNA (B) and m<sup>7</sup>GpppA-RNA (C) were incubated with WNV MTase for assaying N-7 and 2'-O methylations, respectively. The reaction products were digested by nuclease P1 and analyzed on TLC plates. The positions of the origin and the migrations of the G\*pppA, m<sup>7</sup>G\*pppA, and m<sup>7</sup>G\*pppAm molecules are indicated.

the plasmid RNA was incubated with any of the four flavivirus MTases (Fig. 1D). These results demonstrate that flavivirus MTases specifically methylate viral RNA cap structures with a preference for their cognate RNA. The cross-methylations of viral RNA cap, but not nonviral plasmid RNA cap, by the four flavivirus MTases strongly suggest that the conserved 5'-terminal RNA structure and/or sequence of viral genome contain elements critical for the methylation activities.

**RNA elements required for N-7 methylation of WNV cap.** To further dissect the role of RNA elements in flavivirus cap

methylation, we prepared four chimeric RNA substrates, in which the 5'-terminal 4 or 10 nucleotides were interchanged between the WNV RNA and the plasmid pUC RNA (Fig. 2A). Methylation assays with the four chimeric RNAs, together with the pUC RNA, showed no detectable N-7 MTase activity (Fig. 2B), whereas an efficient N-7 methylation of G\*pppA to m<sup>7</sup>G\*pppA was observed for wild-type WNV RNA. These results suggest that the first 10 nucleotides of the viral genome are necessary, but not sufficient, for the N-7 methylation of the WNV cap.

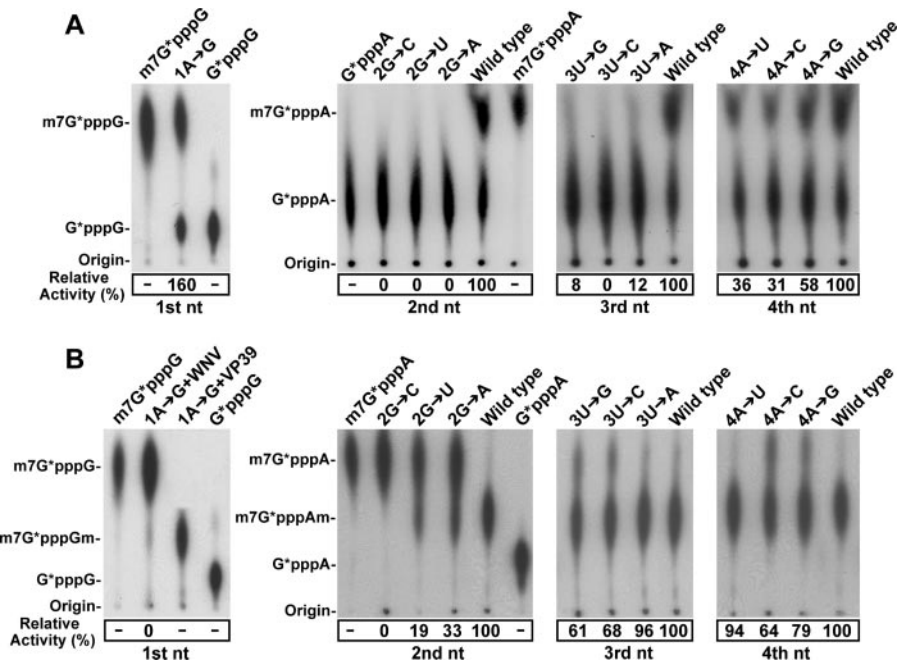


FIG. 3. Analysis of the effect of the first four nucleotides of WNV genome on cap methylation. Each of the first four nucleotides of the WNV genome was mutated to identify nucleotides critical for N-7 (A) and 2'-O (B) methylations. All mutants were prepared in the context of WNV RNA representing the 5'-terminal 190 nucleotides of the genome. For the first nucleotide, only a 1A→G mutant was tested because T7 polymerase could only efficiently initiate with a GTP or ATP (10). For the second, third, and fourth positions, each nucleotide was mutated to three alternative bases. The methylation efficiency of mutant RNAs was compared to the wild-type RNA (set at 100%) and is indicated below the TLC results. The migration positions of the <sup>32</sup>P-labeled G\*pppG and m<sup>7</sup>G\*pppG on TLC plates are identical to the equivalent cold markers (results not shown). The mobility of m<sup>7</sup>G\*pppGm on TLC plates was assigned based on the reaction in which the m<sup>7</sup>G\*pppG-RNA was methylated to m<sup>7</sup>G\*pppGm-RNA by the vaccinia virus VP39.

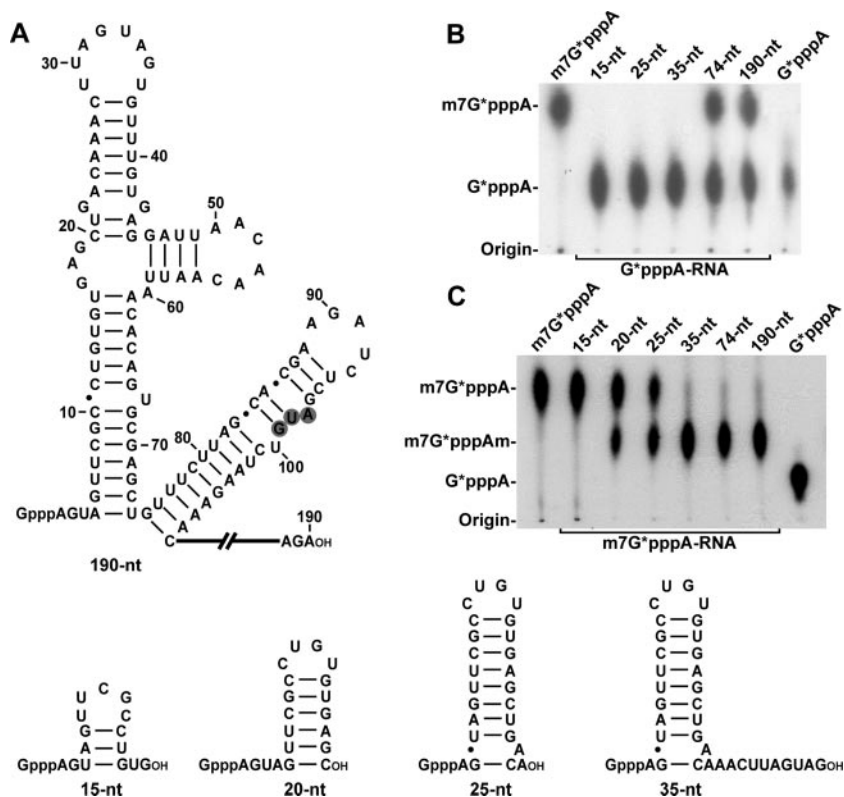


FIG. 4. RNA length requirement for WNV cap methylations. (A) Thermodynamically predicted stem-loop structures formed by various lengths of 5'-terminal WNV genome. The top panel shows the stem-loop structure formed by the 5' UTR of the WNV genome. The nucleotide (nt) positions are numbered. The initiation codon (AUG) of the open reading frame is shaded. The bottom panel presents the MFold-predicted secondary structures formed by the 5'-terminal 15, 20, 25, and 35 nucleotides of the WNV genome. (B) Minimal RNA length essential for N-7 methylation. A set of truncated RNAs containing the 5'-terminal 15, 25, 35, and 74 nucleotides of the WNV genome were prepared; each RNA contained a G\*pppA-cap, and was assayed for N-7 methylation. (C) RNA length requirement for 2'-O methylation. m<sup>7</sup>G\*pppA-RNAs of the indicated lengths were assayed for 2'-O methylation. All methylation reaction products were digested with nuclease P1 and analyzed on TLC plates.

To identify nucleotides of the 5'-terminal region of the WNV RNA critical for N-7 methylation, we mutated each of the first four nucleotides to other possible residues. For the first base, only a 1A→G change was examined, because the T7 RNA polymerase used for *in vitro* transcription could only efficiently initiate transcription with a GTP or ATP (10). For the second, third, and fourth bases, RNAs with all three alternative nucleotides were tested. Substitutions at position 1 did not reduce N-7 methylation, whereas substitutions at positions 2, 3, and 4 were detrimental to N-7 methylation (Fig. 3A). The results demonstrate that, among the first four nucleotides, the identities of the second and third are most critical for type 0 cap formation.

We next examined the minimal length of WNV 5' RNA required for WNV N-7 methylation. Based on the thermodynamically predicted RNA structure, we truncated the RNA so as to retain the 5'-terminal 74, 35, 25, or 15 nucleotides. Among these, the 74-nucleotide RNA could adopt an intact stem-loop structure, whereas the 35-, 25-, and 15-nucleotide RNAs contained only the 5' portion of the stem (Fig. 4A). The 74-nucleotide RNA was an efficient substrate for N-7 methylation; none of the shorter RNAs was methylated by the enzyme (Fig. 4B). The results suggest that the 5' stem-loop structure of the WNV genome is required for N-7 cap methylation.

To identify secondary structural elements within the 5' stem-loop essential for N-7 methylation, we systematically mutated the 74-nucleotide stem-loop structure. We initially performed the mutagenesis in the context of RNA representing the first 190 nucleotides of the WNV genome. As shown in Fig. 5A, the four helices were individually disrupted by a strand replacement (Ha1, Hb1, Hc1, and Hd1); the two loop sequences were individually reversed (La1 and Lb1). Ha1 and Hb1 could not be N-7 methylated, whereas Hc1 and Hd1 were N-7 methylated to 28 and 85% of the wild-type levels, respectively (Fig. 5B). The loop mutants La1 and Lb1, respectively, showed 120 and 88% of the wild-type RNA activity. These results suggest that (i) the bottom two helices (Ha and Hb) are essential for N-7 methylation; (ii) the top helix (Hc) is important, but not essential, for the N-7 MTase activity; and (iii) the side helix (Hd) and the two loop sequences (La and Lb) are not critical for the N-7 methylation.

We restored each of the three critical helices with double-strand replacement by their reverse complementary sequences (Ha2, Hb2, and Hc2; Fig. 5A). The sequence of the restored stem was flipped to examine, besides helix structure, whether the nucleotide identity within each stem is important for methylation activity. Remarkably, these RNAs became efficient substrates for N-7 cap methylation (Fig. 5B). In addition, a dele-

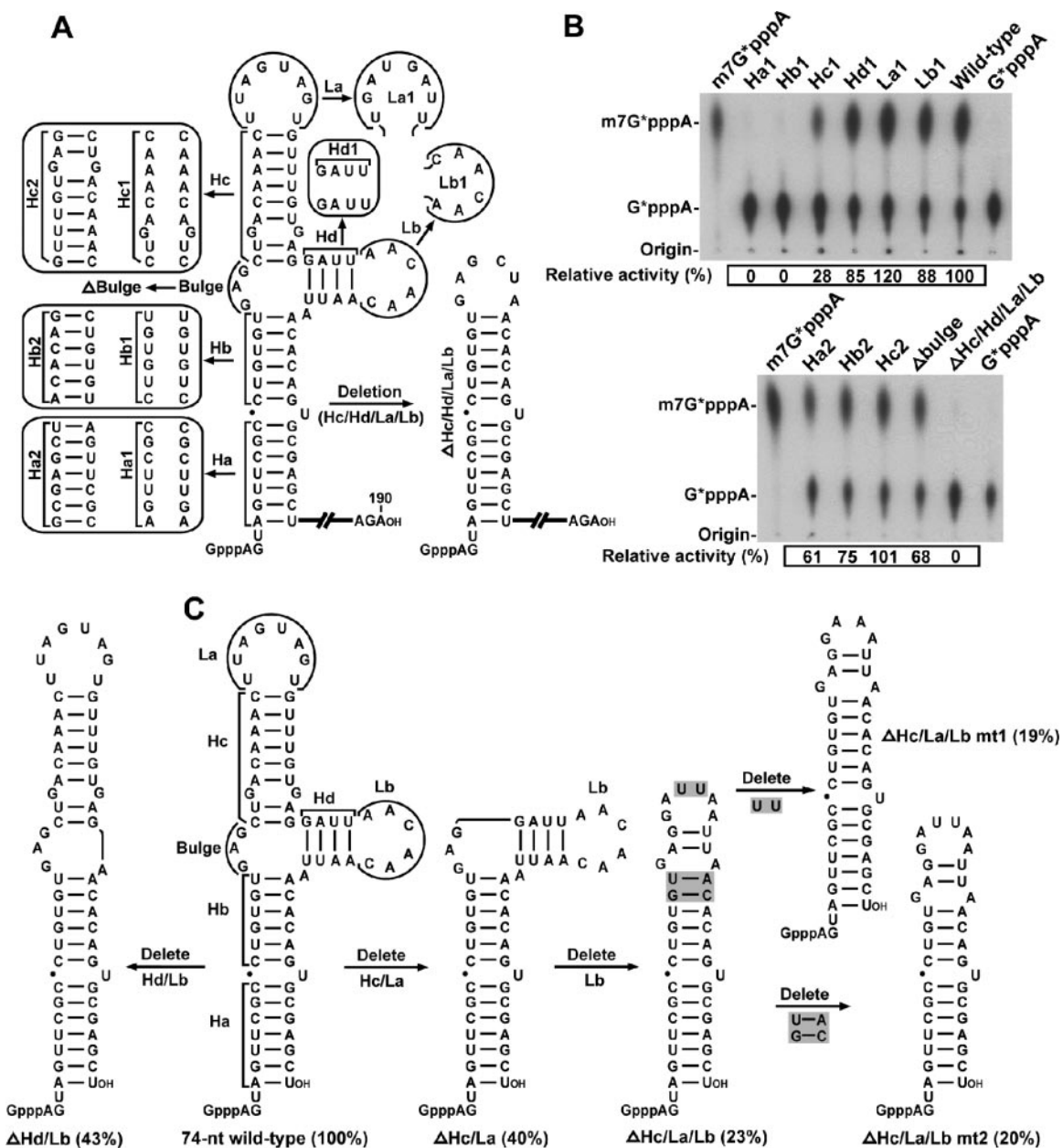


FIG. 5. RNA secondary structure requirement for N-7 methylation of WNV cap. (A) Mutant RNA substrates. The 5'-terminal stem-loop structure of WNV genome contains three helices (Ha, Hb, and Hc), two loops (La and Lb), and a three-nucleotide bulge. Two mutants were prepared for the helices: (i) the double-stranded stem was opened up by strand replacement to its complement (Ha1, Hb1, Hc1, and Hd1), and (ii) the helix structures were restored with double-strand replacement by their respective reverse complementary sequences (Ha2, Hb2, and Hc2). Each of the two loop sequences was reversed (La1 and Lb1). Two deletion mutants were also prepared: one contained the three-nucleotide bulge deletion ( $\Delta$ Bulge); the other contained a deletion of the top and side helices and the two loops ( $\Delta$ Hc/Hd/La/Lb). All mutant RNAs in this panel were constructed in the context of the 5'-terminal 190 nucleotides of WNV genome. (B) N-7 methylation of mutant RNAs indicated in panel A. The methylation efficiency of mutant RNAs was compared to that of the wild-type RNA (set at 100%), and the percentages are indicated below the TLC results. (C) Minimal stem-loop RNA required for N-7 methylation of cap structure. The stem-loop RNA formed by the first 74 nucleotides of the WNV genome (74-nt wild-type), which is a fully active substrate for N-7 cap methylation (Fig. 4B), is truncated to define the minimal stem-loop structure important for N-7 methylation. The rationale for RNA truncation is described in Results, and the RNA secondary structures were predicted by using the Mfold program. Mutant RNAs were assayed for N-7 methylation, the methylation efficiency was compared to that of the 74-nt wild-type RNA (set at 100%), and percentages are indicated in parentheses for each mutant RNA. The average values of two independent experiments are presented.

tion of the three-nucleotide bulge from position 17 to 19 (5'-GAG-3') had little effect on N-7 methylation ( $\Delta$ Bulge; Fig. 5B). The  $\Delta$ Bulge RNA was tested because RNA footprinting results (see below in Fig. 6) showed the bulge region to be protected by NS5 protein.

To determine the minimal stem-loop RNA required for N-7 methylation, we deleted the top and side helices and the two loops (Fig. 5A). The resulting RNA,  $\Delta$ Hc/Hd/La/Lb, was completely inactive for N-7 methylation (Fig. 5B), indicating that part of the deleted region was required for the activity. Since

the stem-loop RNA formed by the first 74 nucleotides (nt) of WNV genome (Fig. 5C, 74-nt wild type) is a fully active substrate for N-7 cap methylation (Fig. 4B), we used the 74-nucleotide RNA to determine which portion within the  $\Delta$ Hc/Hd/La/Lb was required for the activity. As shown in Fig. 5C, mutant RNAs were predicted for the stem-loop structures by the MFold program and assayed for N-7 methylation (TLC images not shown). Deletion of Hd/Lb, Hc/La, or Hc/La/Lb decreased the N-7 methylation to 43, 40, and 23% of the 74-nucleotide wild-type RNA, respectively. Further deletion of  $\Delta$ Hc/La/Lb RNA (2 Us in loop,  $\Delta$ Hc/La/Lb mt1; 2 bp in stem,  $\Delta$ Hc/La/Lb mt2; Fig. 5C) reduced the N-7 activity to 19 and 20% of the 74-nucleotide wild-type RNA, respectively. Overall, the results demonstrate that the RNA structure with greater than 13 bp, but not necessarily the sequence of 5' stem-loop, is critical for N-7 methylation, along with specific nucleotides at the second and third positions.

**RNA elements required for 2'-O methylation of WNV cap.** A similar approach was used to define the RNA elements required for 2'-O ribose methylation. Replacement of the first 4 or 10 nucleotides with the pUC sequence completely abolished 2'-O ribose methylation of the WNV RNA (pUC4-WNV and pUC10-WNV, Fig. 2C). Strikingly, replacement of the first 4 or 10 nucleotides with the WNV sequence conferred efficient 2'-O ribose methylation of the plasmid RNA (WNV4-pUC and WNV10-pUC, Fig. 2C). The results demonstrate that the first four nucleotides of the WNV genome are determinants of efficient 2'-O ribose methylation.

Individual bases within the first four nucleotides of WNV RNA were mutated to other possible residues (Fig. 3B). As discussed earlier, the first nucleotide A was only mutated to a G; no 2'-O ribose methylation was detected with the 1A→G transition. As a positive control, the vaccinia virus VP39 protein, a well-characterized m<sup>7</sup>G\*pppA/G-dependent ribose 2'-O MTase (3), was capable of methylating m<sup>7</sup>G\*pppG-RNA to m<sup>7</sup>G\*pppGm-RNA (Fig. 3B, left panel). For the second nucleotide G, substitution with a U or A, respectively, allowed for 19 or 33% of the wild-type 2'-O methylation, whereas replacement with a C abolished the ribose 2'-O methylation. In contrast to the first two nucleotides, the third and fourth nucleotides could be substituted with less dramatic effect on the 2'-O methylation (Fig. 3B). The mutagenesis results suggest that, among the first four nucleotides, only the first residue A and the second nucleotide G of the RNA substrate are critical for 2'-O methylation.

To determine the minimal RNA chain-length required for 2'-O ribose methylation, we examined RNAs representing the 5'-terminal 15, 20, 25, 35, or 74 nucleotides of the WNV genome (Fig. 4A). A similar extent of 2'-O ribose methylation was observed for the 35-, 74-, and 190-nucleotide RNAs, while methylation was reduced for the 20- and 25-nucleotide RNAs. No 2'-O methylation was detected for the 15-nucleotide RNA. Analysis with an MFold program indicated that each of the above truncated RNAs could potentially form a stem-loop structure (Fig. 4A). To exclude the possibility that secondary structural elements had contributed to the 2'-O methylation, we prepared a set of RNAs containing the 5'-terminal four nucleotides of the viral sequence (GpppAGUA) followed by a (A)<sub>6</sub>, (A)<sub>11</sub>, (A)<sub>16</sub>, (A)<sub>21</sub>, or (A)<sub>31</sub> tail. Methylation assays showed that, compared to the viral 190-nucleotide RNA, the

(A)<sub>6</sub>, (A)<sub>11</sub>, and (A)<sub>16</sub>-containing RNAs retained 22, 47, and 60%, respectively, of the 2'-O methylation; the (A)<sub>21</sub>- and (A)<sub>31</sub>-containing RNAs showed 100% of the 2'-O methylation activity. These results suggest that RNA structure is not required for the 2'-O activity; on the contrary, RNA structure formed by viral sequence may potentially inhibit the 2'-O methylation. In summary, the results demonstrate that an optimal RNA for WNV cap 2'-O methylation requires a minimum length of 20 nucleotides of viral sequence, with a preference for adenine and guanine at the first and second positions, respectively.

**Cap analogues are not active substrates for WNV MTase.** To further demonstrate the specificity of WNV cap methylations, we performed methylation assays using cap analogues, GpppA and m<sup>7</sup>GpppA. Compared to the RNA-based assay, cap analogues allow for testing of high concentrations of substrates, enabling detection of weak activities. Neither N-7 nor 2'-O methylation was detected when unlabeled cap analogues (100  $\mu$ M) were incubated with WNV MTase (2  $\mu$ g) and <sup>14</sup>C-labeled SAM (100  $\mu$ M) (data not shown). As a positive control, recombinant yeast guanine N-7 MTase (Abd1; 0.6  $\mu$ g) (19) efficiently converted GpppA to <sup>14</sup>C-labeled m<sup>7</sup>GpppA (data not shown). Titration of the assay conditions (e.g., pH) did not result in any methylation of the cap analogue (data not shown). Furthermore, cap analogues (up to 10  $\mu$ M tested concentration) could not inhibit cap methylations of the viral 190-nucleotide RNA (data not shown). These results again demonstrate that the RNA chain-length is critical for flavivirus cap methylation.

**Footprinting of WNV RNA-NS5 interactions during N-7 and 2'-O methylations.** RNA footprinting experiments were performed to probe protein-viral RNA interactions prior to the N-7 and 2'-O methylations. Full-length NS5 was incubated with <sup>32</sup>P-cap-labeled G\*pppA-RNA representing the first 190 nucleotides of the WNV genome. The reaction mixture was then digested with RNase I (which cleaves RNA without base specificity) and analyzed on a 20% denaturing PAGE (Fig. 6B). In addition, G\*pppA-RNA was either hydrolyzed in an alkali buffer or digested with RNase T<sub>1</sub> (which cleaves specifically after nucleotide G) to produce an RNA ladder or G ladder, respectively. Comparison of the RNase I digestion pattern between the RNA alone and the RNA-NS5 complex showed that the first three nucleotides (GpppAGU) and nucleotides 18 to 21 (5'-AGCU-3') were protected. The results suggest that NS5 interacts with the 5' cap and a bulge region in the stem-loop structure prior to N-7 methylation.

We then performed footprinting using the m<sup>7</sup>G\*pppA-RNA substrate. The footprinting pattern obtained using m<sup>7</sup>G\*pppA-RNA was similar to that observed with G\*pppA-RNA, except that (i) an extra nucleotide of the 5'-terminal sequence (m<sup>7</sup>G\*pppAGUA) was weakly protected in the m<sup>7</sup>G\*pppA-RNA and (ii) the 5'-AGCU-3' of nucleotides 18 to 21 from m<sup>7</sup>G\*pppA-RNA were less well protected than those from G\*pppA-RNA (compare Fig. 6C with 6B). In this experiment, an extra band, which migrated immediately above molecule m<sup>7</sup>GpppA, was observed (Fig. 6B); this band probably represented the residual [ $\alpha$ -<sup>32</sup>P]GTP that had been used to label the m<sup>7</sup>G\*pppA-RNA and was not completely removed during RNA preparation. We also analyzed the footprinting reactions on a lower-percentage (7%) PAGE to search for potential

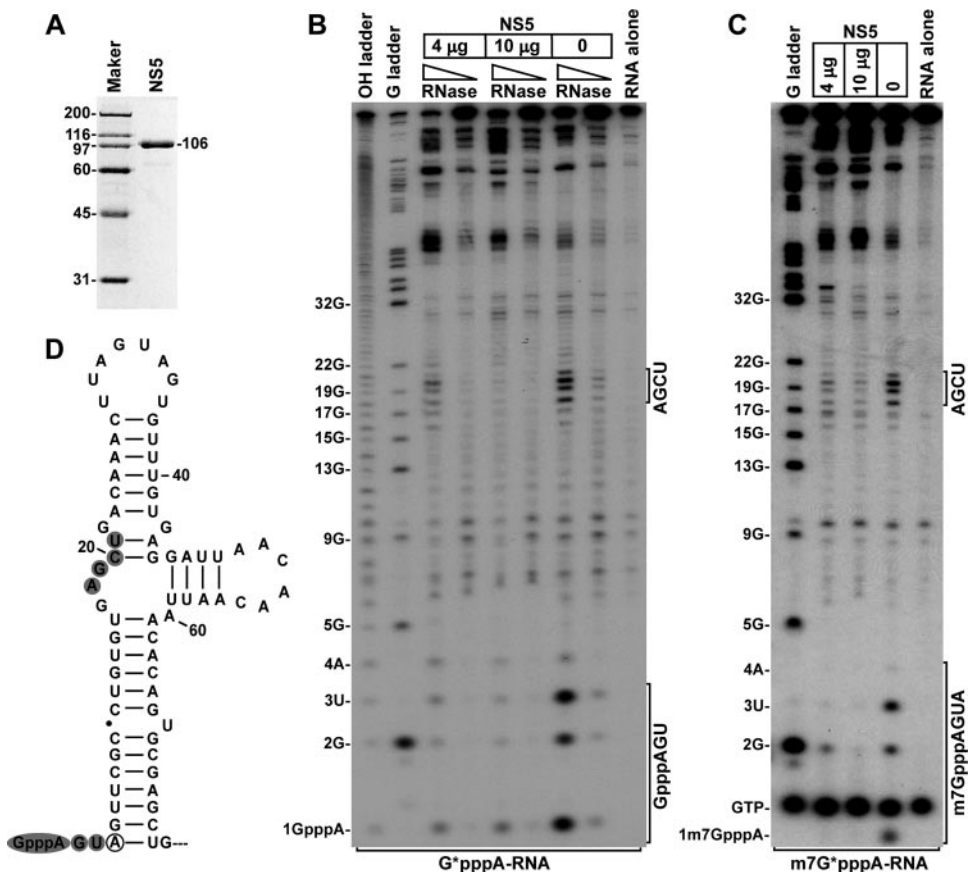


FIG. 6. Footprinting of RNA-NS5 interaction during N-7 and 2'-O methylations. (A) Recombinant full-length NS5 of WNV. The protein was analyzed on an SDS-PAGE stained with Coomassie blue. (B) Footprinting during N-7 methylation. The 5'-labeled G\*pppA-RNA (5'-terminal 190 nucleotides of WNV genome) was incubated with or without NS5 protein, digested with RNase I, and analyzed on a 20% denaturing PAGE. Two concentrations of RNase I (1 or 0.5 U per reaction) were used to ensure limited digestions of RNA as indicated. The G\*pppA-RNA was also hydrolyzed to yield a RNA ladder (OH ladder) digested with RNase T<sub>1</sub> to produce a G ladder. The G\*pppA-RNA without treatment was included as a negative control (RNA alone). The nucleotide positions of the first five residues and the downstream G nucleotides are selectively indicated on the left side. The protected RNA regions are bracketed on the right side. (C) Footprinting during 2'-O methylation. The 5'-labeled m<sup>7</sup>G\*pppA-RNA of WNV was incubated with or without NS5, digested with a single dose of RNase I (1 U per reaction), and analyzed on a denaturing gel. (D) Summary of footprinting. The protected regions during both N-7 and 2'-O methylations are indicated as shaded nucleotides. The extra weakly protected nucleotide after N-7 methylation is circled (residue A at position 4).

downstream protections in the RNA; no further protections were observed (data not shown). Overall, the footprinting results suggest that the 5' terminus of RNA specifically interacts with NS5 during the N-7 and 2'-O cap methylations.

**Inhibition of cap methylations by an antisense oligomer targeting the 5' terminus of WNV RNA.** To further investigate the role of the 5' terminus of the viral genome in cap methylation, we tested whether an antisense oligomer (5' end), which hybridizes with the 5'-terminal 20 nucleotides of the WNV genome, could inhibit NS5 MTase activity in vitro. The oligomer contained a modified backbone, namely, PMO, which is uncharged, water soluble, and nuclease resistant (28). This 5'-end PMO is a potent inhibitor of WNV in cell culture (11). As expected, the 5'-end PMO suppressed both N-7 cap and 2'-O ribose methylations in a dose-responsive manner (Fig. 7). The N-7 methylation was less sensitive to the 5' PMO suppression than was the 2'-O methylation. As a negative control, a scrambled PMO, which contained a randomized sequence with the same base composition as the 5'-end PMO, did not show

inhibition at the equivalent concentrations. These results further support the conclusion that the 5' terminus of the viral genome contains signals critical for WNV cap methylation.

DISCUSSION

Our recent studies (25, 33) suggest that flavivirus NS5 represents a unique system to study RNA cap methylation: (i) a single MTase domain catalyzes two distinct methylation reactions; (ii) the N-7 cap methylation precedes the 2'-O ribose methylation; (iii) the 2'-O ribose methylation can be uncoupled from the N-7 cap methylation (i.e., m<sup>7</sup>GpppA-RNA can be readily methylated to m<sup>7</sup>GpppAm-RNA); and (iv) the enzyme specifically methylates the cap on the viral RNA. The RNA substrate specificity during flavivirus cap methylations suggests that the 5'-terminal bases of viral RNA interact with MTase residues. This would be in contrast to cellular and other MTases, which methylate the cap structure in an RNA sequence-nonspecific manner. In the well-studied VP39, the en-



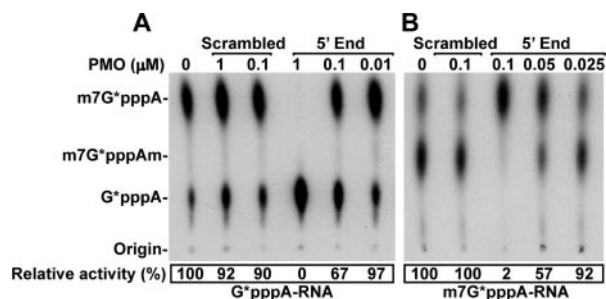


FIG. 7. Inhibition of methylation by an antisense oligomer targeting the 5' terminus of the WNV genome. Two PMOs were synthesized: one contained a sequence complementary to the first 20 nucleotides of WNV genome (5' end), and the other had a randomized sequence (scrambled). The scrambled or 5'-end PMO was incubated with WNV RNA (5'-terminal 190 nucleotides) at the indicated concentrations and was assayed for its effects on N-7 (A) and 2'-O (B) methylations. G\*pppA-RNA and m<sup>7</sup>G\*pppA-RNA substrates were used for the N-7 and 2'-O methylation assays, respectively. The methylation reactions were digested by nuclease P1, analyzed on TLC plates, and quantified by using a PhosphorImager. The effects of PMOs on cap methylation were indicated by the relative activities with respect to the methylations without PMO treatment (set as 100%). The migration positions of G\*pppA, m<sup>7</sup>G\*pppA, m<sup>7</sup>G\*pppAm, and the origin are indicated on the left side of the TLC plates. One result representative of two independent experiments is shown.

zyme was found to solely interact with the sugar-phosphate backbone of a RNA substrate in a crystal structure (16). In line with our results, specific N-7 cap methylation was recently reported for Sendai virus L protein (23). Recombinant L protein preferentially methylates capped RNA with viral mRNA 5' sequences (GpppAGG); however, the specificity of 2'-O ribose methylation could not be analyzed because the recombinant protein does not possess a 2'-O methylation activity (23).

MTases from DENV-1, YFV, WNV, and PWV methylate the cap structure at both the N-7 and 2'-O positions of WNV or PWV RNA (Fig. 1). The cross-methylation activities among different flaviviruses could result from the conservation of 5' RNA structure and 5'-terminal nucleotides of the four tested viruses. Indeed, a conserved stem-loop structure, similar to that presented for WNV (Fig. 4A), was thermodynamically predicted for the 5' UTRs of DENV-1, YFV, and PWV (data not shown). Sequence alignment showed that WNV and YFV have an identical 5' four nucleotides (GpppAGUA), DENV-1 has a one-nucleotide difference at the fourth position (underlined; GpppAGUU), and PWV has a two-nucleotide difference at the third and fourth positions (underlined; GpppAGAU). In contrast, an RNA derived from plasmid pUC19 is not a substrate for flavivirus MTase because it contains a 5'-terminal sequence that is different except for the first A residue (Fig. 2A) and does not form the 5' stem-loop structure that is conserved in flavivirus RNA. In agreement with the sequence analysis, mutagenesis results demonstrate that specific nucleotides within the first four positions of RNA substrate are important for WNV cap methylations (Fig. 3).

Using WNV as a model, we have defined the RNA elements required for cap methylations by NS5. The first four nucleotides of the viral genome were required for both N-7 and 2'-O methylations, among which the second and third nucleotides

are most important for N-7 cap methylation, whereas the first and second nucleotides are most critical for 2'-O ribose methylation (Fig. 3). In addition, the 5' stem-loop structure is required for the N-7 methylation. Notably, the sequence of the stem-loop appears to be less important than the secondary structure itself, given that reversal of the loop sequences and restoration of the helices with their reverse complementary strand did not affect the N-7 methylation. In contrast, the 2'-O ribose methylation does not require the 5' stem-loop structure but does need a minimum viral RNA of 20 nucleotides. Footprinting experiments showed that, during both methylations, WNV NS5 protein protects the first three nucleotides, as well as a bulge region spanning nucleotides 18 to 21 of the viral RNA. However, in the context of 5' 190-nucleotide WNV RNA, the protected bulge region does not appear to be important for cap methylation, given that bulge-deleted RNA could be efficiently methylated (Fig. 5). Since both methylation events require the RNA substrates to be longer than 20 nucleotides, these nucleotides may function as a docking site for enzyme binding. We expect that the specific interactions defined for the WNV model will be applicable to other flavivirus cap methylations; however, divergence from this model due to evolutionary distances among various flaviviruses cannot be ruled out.

One striking feature of flavivirus MTases is that they catalyze two methylations at different positions of the cap structure by utilizing only a single SAM-binding site, as demonstrated by crystal structures of MTase from DENV (12) and WNV (33). These results led us to hypothesize that the 5' terminus of the RNA must be repositioned in order to accept the methyl group from SAM during the two methylation events: the guanine N-7 would first be positioned in proximity to the methyl group of SAM to generate m<sup>7</sup>GpppA-RNA; after N-7 methylation, the m<sup>7</sup>Gppp moiety moves into the GTP-binding pocket, as previously described in the DENV-2 MTase structure (12); the binding of m<sup>7</sup>Gppp moiety moves the ribose 2'-OH of the first transcribed adenosine in close proximity to SAM to accept the 2'-O methyl group. This model is supported by the footprinting results, in which the first three and four nucleotides of Gppp-RNA and m<sup>7</sup>GpppRNA were, respectively, protected by NS5, and the protection of 5'-AGCU-3' of nucleotides 18 to 21 became weaker after the N-7 methylation. Cocystal structures of MTase and RNAs with or without N-7 methylation are ultimately needed to illuminate the specific interactions.

The specificity of flavivirus cap methylation has provided an opportunity to search for inhibitors that selectively block flavivirus cap methylations without affecting host mRNA methylations. We recently showed that NS5 mutations abolishing both the N-7 and the 2'-O methylations are lethal for WNV replication in cell culture (25, 33). Therefore, compounds that specifically inhibit flavivirus cap methylation could potentially be developed for antiviral therapy. The conserved interactions between viral RNA and NS5 during cap methylations may enable the identification of small molecules that inhibit a broad spectrum of flaviviruses. In support of this hypothesis, we previously showed that an antisense PMO complementary to the 5'-terminal 20 nucleotides of WNV genome potently inhibited viral production in cell culture: the PMO reduced virus titers by 5 to 6 logs at a 5

$\mu\text{M}$  concentration without cytotoxicity (11). Furthermore, this PMO could partially protect mice from WNV disease (P.-Y. Shi et al., unpublished results). In the present study, we have demonstrated that the 5'-end PMO can inhibit cap methylations. We currently do not know what caused the differing sensitivities of the N-7 and 2'-O methylations to the 5'-end PMO inhibition (Fig. 7).

Viruses represent an attractive system for studying the molecular basis of RNA capping and its relationship to RNA transcription and replication. Although the guanylyltransferase for flavivirus capping remains elusive, our results indicate that cap methylation may specifically target plus-sense RNA during flavivirus replication. Viral minus-sense RNA is hybridized with the plus-sense template in the replication form or replication intermediate (8, 9). Even if capped, the 5' terminus of minus-sense RNA lacks the structural signals required for N-7 methylation and, therefore, would not be methylated. On the other hand, multiple rounds of plus-sense RNA are simultaneously synthesized from the minus-sense RNA template. Since the MTase and RdRp are physically linked within a single NS5 protein, the plus-sense RNA capping and methylation are likely coupled to nascent RNA synthesis. Capping of cellular RNA is targeted to the transcript made by RNA polymerase II through direct binding of the capping apparatus to the RNA polymerase II elongation complex (7, 20, 32). The RdRp domain of DENV-2 was recently shown to recognize the 5'-terminal elements of the viral genome to promote RNA synthesis (13). Experiments are needed to examine the interactions between the flavivirus capping and RNA replication.

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