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# Structural and Functional Analysis of Methylation and 5′-RNA Sequence Requirements of Short Capped RNAs by the Methyltransferase Domain of Dengue Virus NS5

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The N-terminal 33 kDa domain of non-structural protein 5 (NS5) of dengue virus (DV), named $NS5MTase_{DV}$, is involved in two of four steps required for the formation of the viral mRNA cap $^{7Me}GpppA_{2'OMe}$, the guanine-N7 and the adenosine-2′O methylation. Its *S*-adenosyl-L-methionine (AdoMet) dependent 2′O-methyltransferase (MTase) activity has been shown on capped $^{7Me\pm}GpppAC_n$ RNAs. Here we report structural and binding studies using cap analogues and capped RNAs. We have solved five crystal structures at 1.8 Å to 2.8 Å resolution of $NS5MTase_{DV}$ in complex with cap analogues and the co-product of methylation *S*-adenosyl-L-homocysteine (AdoHcy). The cap analogues can adopt several conformations. The guanosine moiety of all cap analogues occupies a GTP–binding site identified earlier, indicating that GTP and cap share the same binding site. Accordingly, we show that binding of $^{7Me}GpppAC_4$ and $^{7Me}GpppAC_5$ RNAs is inhibited in the presence of GTP, $^{7Me}GTP$ and $^{7Me}GpppA$ but not by ATP. This particular position of the cap is in accordance with the 2′*O*-methylation step. A model was generated of a ternary 2′*O*-methylation complex of $NS5MTase_{DV}$, $^{7Me}GpppA$ and AdoMet. RNA-binding increased when $^{7Me\pm}GpppAGC_{n-1}$ starting with the consensus sequence GpppAG, was used instead of $^{7Me\pm}GpppAC_n$. In the $NS5MTase_{DV}$–GpppA complex the cap analogue adopts a folded, stacked conformation uniquely possible when adenine is the first transcribed nucleotide at the 5′ end of nascent RNA, as it is the case in all flaviviruses. This conformation cannot be a functional intermediate of methylation, since both the guanine-N7 and adenosine-2′O positions are too far away from AdoMet. We hypothesize that this conformation mimics the reaction product of a yet-to-be-demonstrated guanylyltransferase activity. A putative *Flavivirus* RNA capping pathway is proposed combining the different steps where the NS5MTase domain is involved.



*Keywords:* methyltransferase; capping; flavivirus; dengue; guanylyltransferase

## Introduction

Despite a wide diversity in genome organization and replication mechanism, many eukaryotic cellular and viral RNAs are modified by addition of a cap structure consisting of a guanine connected through an 5′–5′ triphosphate bridge to the first transcribed nucleoside and methylated at the N7 position. This cap 0 structure ($^{7Me}GpppN$…) is often converted in a cap 1 structure ($^{7Me}GpppN_{2'OMe}$…) by methylation of the 2′-O position of the first nucleotide. The main role of the cap structure is to protect mRNA from degradation by 5′ exoribonucleases and to enhance

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Abbreviations used: DV, dengue virus; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; RTPase, RNA triphosphatase; GTase, guanylyltransferase; 2′OMTase, nucleoside-2′-O-methyltransferase; N7MTase, guanine-N7-methyltransferase; NS, non-structural protein.

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initiation of mRNA translation.[1–3] The interest in viral capping is reinforced by the fact that inhibiting the cap formation may prevent viral replication.[4–7] Consequently, structural and biochemical work deciphering viral mRNA capping mechanisms may add some other names than that of proteases and polymerases to the enzyme target list for antiviral drug research.

In eukaryotic cells, the cap is added co-transcriptionally in the nucleus on nascent transcripts and is completed by three sequential enzymatic activities[1,3]: (i) an RNA triphosphatase (RTPase) removes the 5′ γ-phosphate group of mRNA; (ii) a guanylyltransferase (GTase), or capping enzyme, catalyzes the transfer of GMP to the remaining 5′-diphosphate end of mRNA; and (iii) a *S*-adenosyl-L-methionine (AdoMet)-dependent (guanine-N7)-methyltransferase (N7MTase) methylates the cap at the N7 position. Whereas lower eukaryotes including yeast contain a cap 0 structure, in higher eukaryotes a nuclear AdoMet-dependent (nucleoside-2′-O-)-methyltransferase (2′OMTase) completes the cap 1 structure. In the case of viruses, which replicate in the cytoplasm and code for their own RNA capping machinery, cap formation may follow the sequential three or four-step strategy of eukaryotic mRNA cap formation. Nonetheless, some of them have acquired specific capping strategies, as shown for alphaviruses that methylate GTP prior to the transfer of $^{7Me}$GMP to the 5′-diphosphate end of the RNA.[8] Another example is the negative-sense (−) single-stranded (ss) RNA vesicular stomatitis virus, which transfers GDP rather than GMP onto the 5′-monophosphate end of RNA.[9] In addition to viruses replicating in the cytoplasm, some replicate in the nucleus and acquire their RNA cap by hijacking the host cell capping apparatus (i.e. positive-sense (+) ssRNA retroviruses such as HIV[10]), or by stealing a cap structure from cellular mRNAs in a process called cap snatching (i.e. negative strand (-) ssRNA viruses such as influenza virus[11]).

Despite the cap structures being conserved in many organisms, there is a large diversity in the molecular organization of the RNA capping machinery. In metazoans and plants the RTPase and GTase reactions are performed by a single two-domain protein, while the MTase activities reside in two separate single-domain enzymes.[1] In yeast, three separate proteins are each responsible for one of the three catalytic activities. For viral capping machineries a variety of combinations have been described. In the case of the DNA vaccinia virus, one protein bears RTPase, GTase and N7MTase activities on separate domains and another protein bears the 2′OMTase activity.[1] For double-stranded (ds) RNA orthoreovirus, the RTPase activity resides in one domain of either protein λ1[12] or μ2,[13] and the other three activities in three separate domains of protein λ2.[14] For (+) ssRNA alphaviruses the RTPase activity is found on one domain of non-structural protein 2 (nsp2), while the GTase and N7MTase are located on separate domains of protein nsp1.[15]

The (+) ssRNA genome of viruses of the genus *Flavivirus* bears a cap 1 structure $^{7Me}$GpppA$_{2′OMe}$G where the first two nucleotides are strictly conserved.[16] There are over 70 flaviviruses, including important human pathogens, i.e. dengue, yellow fever and West Nile virus. They seem to code for their own capping machinery although it has not yet been entirely established. The multifunctional non-structural protein NS3 was shown to carry RTPase activity within the C-terminal helicase domain.[17–19] The GTase activity has not been identified. The 2′OMTase activity was first demonstrated for the 33 kDa N-terminal domain of dengue virus (DV) protein NS5 (NS5MTase$_{DV}$) using small capped RNA substrates $^{7Me±}$GpppAC$_n$ (meaning both methylated and non-methylated at guanine-N7).[20,21] Recent works demonstrated that the same MTase domain of dengue virus, and also of the cognate West Nile virus (WNV) NS5 and yellow fever virus (YFV), bears both the N7 and the 2′O-MTase activities when longer capped RNAs were used with the 5′ sequence of the WNV genome.[7,22,23] Thus, the *Flavivirus* MTase domain seems to have an active center that is able to conduct a methyl transfer reaction onto two distinct acceptor positions, which are rather different in their chemical characteristics and conformational context. The only other example, which has been reported recently, is the MTase domain of the large protein of the (−) ssRNA vesicular stomatitis virus.[24] To accomplish this task the mRNA cap substrate has to be accommodated in two very different positions: one with the guanine-N7 position and the other with the nucleoside-2′O position facing the AdoMet methyl group. Thus, it has to be repositioned between the two methylation steps. The authors also showed that the N7-methyltransfer precedes the 2′O-methyltransfer.[7,22]

The crystal structure of NS5MTase$_{DV}$ was solved with an *S*-adenosyl-L-homocysteine (AdoHcy), the co-product of the methyltransfer, in the AdoMet-binding site.[20] When a non-hydrolyzable GTP analogue was soaked into the crystals, it did not fix in the active site but went exclusively to a second binding pocket at 12 Å from the AdoMet binding site (complex structure deposited under PDB code 2P1D).[20] This site binds selectively GTP and was interpreted as a site that accommodates the cap during the methyltransfer to the nucleoside-2′O position of nascent RNA.

In order to further understand the molecular mechanism of RNA cap binding and methylation by NS5MTase$_{DV}$, we conducted binding and structural studies using cap-analogues and small uncapped or capped RNAs. Here we report the crystal structures of complexes of NS5MTase$_{DV}$ with five different cap analogues. NS5MTase$_{DV}$ accommodated these molecules in three different conformations, two of which are proposed to have relevance in the *Flavivirus* RNA capping pathway. The binding assays with specific 2′O-methylation substrates $^{7Me±}$GpppAC$_n$ showed that NS5MTase$_{DV}$ binds exclusively the capped form and binding