Chapter 10

Messenger RNA Cap Methylation in Vesicular Stomatitis Virus, a Prototype of Non-Segmented Negative-Sense RNA Virus

Jianrong Li and Yu Zhang

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54598

1. Introduction

The non-segmented negative-sense (NNS) RNA viruses encompass a wide range of significant human, animal, and plant pathogens including several National Institute of Allergy and Infectious Diseases (NIAID) Category A and C biodefense pathogens. The NNS RNA viruses are classified into four families: the *Rhabdoviridae,* as exemplified by rabies virus and vesicular stomatitis virus (VSV); the *Paramyxoviridae,* as exemplified by human respiratory syncytial virus (RSV), human metapneumovirus (hMPV), human parainfluenza virus type 3 (PIV3), measles virus, mump virus, Newcastle disease virus (NDV), Nipah virus, and Hendra virus; the *Filoviridae,* as exemplified by Ebola and Marburg viruses; and the *Bornaviridae*, as exemplified by Borna disease virus. For many of these viruses, there are no effective vaccines or anti-viral drugs. RSV, hMPV, and PIV3 account for more than 70% of acute viral respiratory diseases, especially in infants, children, and the elderly [1, 2]. hPIV 1-3 have been recognized as the causative agents of croup since the late 1950's [3]. In addition, measles remains a major killer of children worldwide, despite successful vaccination programs in developed countries [4]. The most virulent strains of NDV, the viscerotropic velogenic strains (often called "exotic" NDV), are classified as High Consequence Livestock Pathogens by USDA due to their potential as agents of agricultural bioterrorism [5].

Messenger RNA modification is the essential issue in NNS RNA virus gene expression and replication. During viral RNA synthesis, NNS RNA viruses produce capped, methylated, and polyadenylated mRNAs [6-8]. Cap formation is essential for mRNA stability, efficient translation, and gene expression [9-11]. It is now firmly established that mRNA capping and methylation in NNS RNA viruses evolves in a mechanism distinct to their hosts [12-19]. Thus, mRNA cap formation is an attractive antiviral target for NNS RNA viruses. For decades, VSV has been

used as a model to understand the replication and gene expression of NNS RNA viruses. Most of our understanding of mRNA modifications of NNS RNA viruses comes from studies of VSV, a prototype of the *Rhabdoviridae* family. Using VSV as a model, we will discuss (i) the unusual mechanism of mRNA capping and cap methylation; (ii) the impact of viral mRNA cap methylation in viral life cycle and viral pathogenesis; and (iii) the applications of viral mRNA cap methylation in the development of novel vaccines and broadly-active anti-viral agents.

2. Overview diagram of VSV mRNA synthesis and modifications

2.1. The structure of VSV virions

VSV virions are bullet-shaped particles 170 nm in length and 80 nm in diameter (Fig.1). Among NNS RNA viruses, VSV has the simplest RNA genome consisting of 11,161 nucleotides (nt) organized into five VSV genes encoding nucleocapsid (N), phospho- (P), matrix (M), glyco- (G), and large (L) proteins, and leader and trailer regulatory sequences arranged in the order 3′-(leader), N, P, M, G, L, (trailer)-5′[20-23]. Like all NNS RNA viruses, the genome is encapsidated with the N protein to form a nuclease-resistant helical N-RNA complex that is the functional template for mRNA synthesis as well as genomic RNA replication. The N-RNA complex is tightly associated with the viral RNA-dependent RNA polymerase (RdRp), which is comprised of the 241-kDa L protein catalytic subunit and the 29-kDa essential P protein cofactor, and results in the assembly of a viral ribonucleoprotein (RNP) complex [24, 25]. This structure contains the minimum virus encoded components of the VSV RNA synthesis machinery [26]. The RNP complex is further surrounded by the M protein which plays a crucial role in virus assembly, budding, and maintenance of the structural integrity of the virus particle [27]. The outer membrane of virion is the envelope composed of a cellular lipid bilayer. The transmembrane G protein is anchored in the viral envelope, which is essential for receptor binding and cell entry [28].

2.2. VSV life cycle

The overview picture of VSV life cycle is depicted in Fig.2. Upon attaching to an unknown cell receptor(s), VSV enters host cells via receptor mediated endocytosis [29]. Following low pH triggered fusion and uncoating, the RNP complex is delivered into the cytoplasm where RNA synthesis and viral replication occur [30]. During primary transcription, the input RdRp recognizes the specific signals in the N-RNA template to transcribe six discrete RNAs: a 47-nucleotide leader RNA (Le+), which is neither capped nor polyadenylated, and 5 mRNAs that are capped and methylated at the 5′ end and polyadenylated at the 3′end. These mature mRNAs are then translated by host ribosomes to yield functional viral proteins which are required for viral genome replication. During replication, the RdRP initiates at the extreme 3′ end of the genome and synthesizes a full-length complementary antigenome, which subsequently serves as template for synthesis of full-length progeny genomes. These progeny genomes can then be utilized as templates for secondary transcription, or assembled into infectious particles. Finally, viral proteins and genomic RNA are assembled into complete virus particles and the virus exits the cell by budding through the plasma membrane.

http://dx.doi.org/10.5772/54598

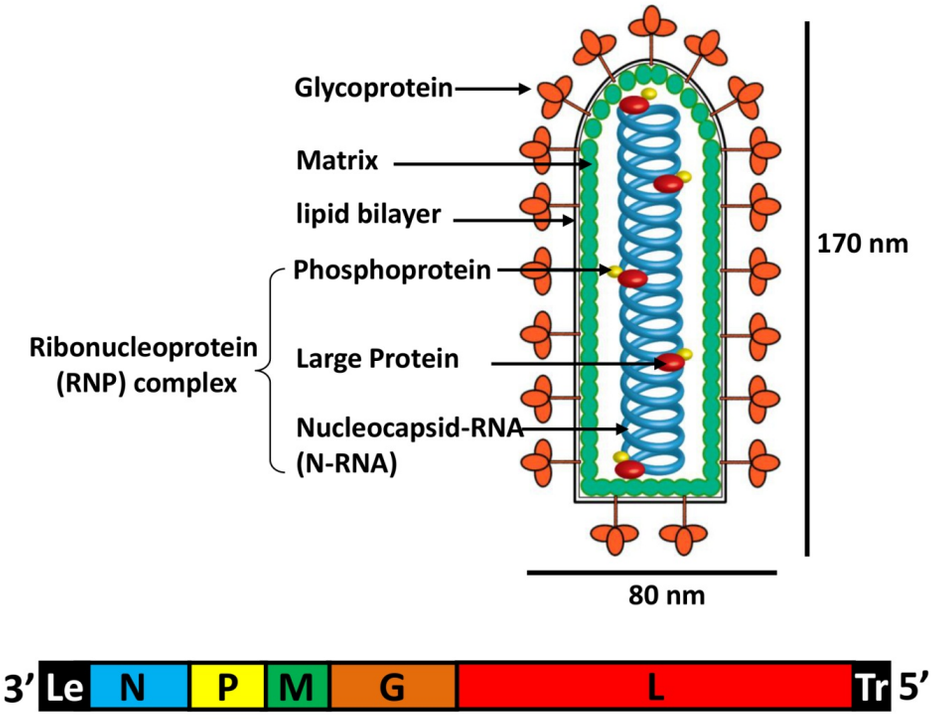

Figure 1. VSV virion structure and genome organization. VSV encodes five structural proteins**:** nucleocapsid (N), phospho- (P), matrix (M), glyco- (G), and large (L) proteins. The VSV genome is arranged in the order 3'-(leader), N, P, M, G, L, (trailer)-5'.

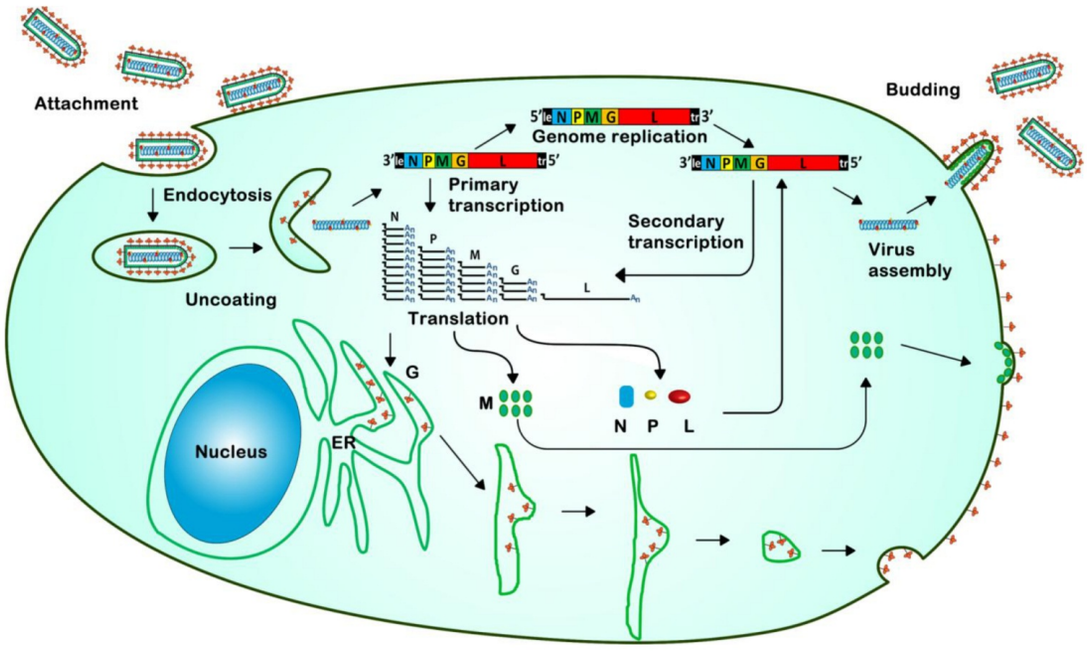

Figure 2. Overview diagram of VSV life cycle. Steps of virus life cycle: attachment, endocytosis, uncoating, genome replication, mRNA transcription, viral protein translation, viral assembly, and budding are shown.

2.3. VSV mRNA synthesis and modifications

Our current understanding of VSV mRNA synthesis and modification can be summarized as follows. In response to a specific promoter element provided by the genomic leader region, the polymerase initiates mRNA synthesis at the first gene-start sequence to synthesize N gene. The nascent mRNA is capped through an unconventional mechanism in which the GDP: polyribonucleotidyltransferase (PRNTase) of L transfers a monophosphate RNA onto a GDP acceptor through a covalent protein-RNA intermediate [12, 13, 16]. Following cap addition, VSV mRNAs are sequentially methylated at ribose 2'-O position and G-N-7 position, which is distinct from all known methylation reactions [17, 18]. Unlike traditional cap forming enzymes, the VSV capping and methylation machinery requires *cis*-acting signals in the RNA [12, 18, 31, 32]. When encountering a gene-end sequence, L polyadenylates and terminates mRNA synthesis by a programmed stuttering of the polymerase on a U7 tract [33, 34]. Termination at the end of the N gene is essential for the polymerase to initiate synthesis at the start of the next gene, to produce the P mRNA. During transcription, the RdRp complex transcribes the viral genome into five mRNAs in a sequential and gradient manner, such that 3' proximal genes are transcribed more abundantly than 3' distal genes [21-23]. This gradient of transcription reflects a poorly understood transcriptional attenuation event that is localized to the gene junction regions. Using this fashion, VSV produces five capped, methylated, and polyadenylated mRNAs, N, P, M, G, and L.

3. Large (L) polymerase protein, the multifunctional protein that modifies viral mRNA

All NNS RNA viruses encode a large (L) polymerase protein, a multifunctional protein ranging from 220-250kDa in molecular weight. The L protein contains enzymatic activities for nucleotide polymerization, mRNA cap addition, cap methylation, and polyadenylation. To date, the structure of L protein, or L protein fragments, has not been determined for any of the NNS RNA viruses. Amino acid sequence alignment between the L proteins of representative members of each family within NNS RNA viruses has identified six conserved regions numbered I to VI (CRs I–VI) (Fig.3) [35]. Thus, there is a general assent that the enzymatic activities of L protein are located in these conserved regions. For the last four decades, VSV L protein has been used a model to understand the different activities of NNS RNA virus L proteins because it is the only member of this order of viruses for which robust transcription can be reconstituted *in vitro* [6, 7, 21, 36]. In addition, the VSV L protein can be highly expressed in recombinant expression systems, such as E.coli and insect cells. The purified VSV L protein retains all the enzymatic activities that can modify short virus-specific mRNA *in trans* [12, 16, 18, 37]. In recent years, many breakthroughs have been made in the characterization of the function of VSV L protein and the enzymatic activities have been mapped at the single amino acid level (Fig.3). Within the primary sequence of L are six conserved regions shared among all NNS RNA virus L proteins. The RdRP activity has been identified in CR III and this region is also required for polyadenylation [38-40]. Consistent with this, a GDN motif is conserved in CR III of all NNS RNA virus L proteins and is functionally equivalent to the GDD polymerization motif characteristic of posi-

http://dx.doi.org/10.5772/54598

tive strand RdRPs. Mutations to the GDN motif of the VSV L protein inactivated polymerase function [40]. The mRNA capping enzyme maps to CR V [13, 16], and the capping activities of L differ from those of other viruses and their eukaryotic hosts. Specifically, an RNA:GDP PRNTase activity present within CR V transfers 5′ monophosphate RNA onto a GDP acceptor through a covalent L-pRNA intermediate [12, 13, 16]. The mRNA cap methyltransferases (MTase) map to CR VI [14, 17, 19]. Like the unconventional capping enzyme, methylation of the VSV mRNA cap structure is also unique in that mRNA cap is modified by a dual specificity MTase activity within CR VI whereby ribose 2′-O methylation precedes and facilitates subsequent guanine-N-7 (G-N-7) methylation [17, 18]. Although functions have not yet to be assigned to the other three conserved regions (CRs I, II, and IV), experiments with Sendai virus (SeV) have implicated CR I in binding P protein and CR II in binding the RNA template [41, 42].

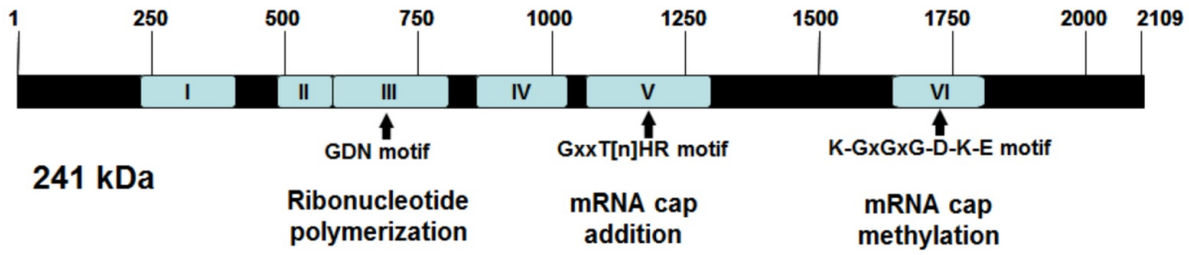

Figure 3. Conserved regions in L proteins of NNS RNA viruses. Six conserved regions numbered I to VI (CRs I–VI) in L protein are shown. Signature motifs for nucleotide polymerization, mRNA cap addition, and cap methylation are shown.

The location of the nucleotide polymerization, capping, and cap methylation activities within separate regions of L has led to the notion that L protein may be organized as a series of independent structural domains. Consistent with this idea, a fragment containing CRs V and VI of the SeV L protein were expressed independently and shown to retain the ability to methylate short RNAs that corresponded to the 5′ end of SeV mRNA [43]. In addition, recombinant VSV and measles virus can be recovered from infectious cDNA clones by inserting the coding sequence of green fluorescent protein between CR V and VI in L gene, suggesting that L protein folds and functions as a series of independent globular domains [44, 45]. Interestingly, mutations to a variable region between CRs V and VI (residues 1450–1481) affect mRNA cap MTase activity, feasibly suggesting that mutation to this hinge region may affect a conformational change in CR VI [46]. More recently, the molecular architecture of VSV L protein has been revealed using negative stain electron microscopy (EM) in combination with proteolytic digestion and deletion mapping [37]. It was found that VSV L protein is organized into a ring domain containing the RNA polymerase and an appendage of three globular domains containing the cap-forming activities. The capping enzyme maps to a globular domain, which is juxtaposed to the ring, and the cap methyltransferase maps to a more distal and flexibly connected globule. Interestingly, upon binding to P protein, L protein undergoes a significant structural rearrangement that may facilitate the coordination between mRNA synthesis and capping apparatus [37, 47].

4. An unconventional mRNA capping mechanism in VSV

4.1. Conventional mechanism of mRNA capping in eukaryotic cells

In eukaryotic cells, capping of mRNA is an early posttranscriptional event that is essential for subsequent processing, nuclear export, stability, and translation of mRNA [11, 48]. Cap formation is mediated by a series of enzymatic reactions (Fig.4A). First, the 5′ triphosphate end of the nascent mRNA chain (5′pppN-RNA) is hydrolyzed by an RNA triphosphatase (RTPase) to yield the diphosphate 5′ ppN-RNA. Second, an RNA guanylyltransferase (GTase) reacts with GTP to form a covalent enzyme-GMP intermediate and transfers GMP to 5′ppN-RNA via a 5′-5′ triphosphate linkage to yield 5′ GpppN-RNA. Typically, RNA GTases contain a signature Kx[D/N]G motif that functions as an active site for the capping reaction [11, 48, 49]. A lysine residue within Kx[D/N]G motif forms the enzyme-GMP covalent intermediate, prior to its transfer onto the diphosphate RNA acceptor [11, 48, 50]. This mRNA capping reaction is conserved among all eukaryotes.

Viruses are highly diverse in capping their mRNA. Many DNA viruses (such as vaccinia virus and baculovirus), double stranded RNA viruses (such as reovirus, rotavirus, and bluetongue virus), and single strand positive RNA viruses (such as West Nile virus, Fig.4B) utilize the conventional eukaryotic capping pathway [51-56]. It has been suggested that Kx[V/L/I]S motif serve as the GTase active site for reovirus and rotavirus [57]. Other viruses have evolved different mechanisms for acquiring their cap. For example, influenza virus and hantavirus furnish their mRNA with this structure by a cap-snatching mechanism, in which the viral polymerase steals host cell mRNA caps to prime viral mRNA synthesis [58, 59]. The alphaviruses, such as Sindbis, have evolved S-adenosyl-L-methionine(SAM)-dependent GTase activities that utilize distinct motifs (such as HxH motif) to transfer 7^{m}Gp through a covalent histidine Gp intermediate to form the 7^{m}GpppN cap [60].

4.2. An unconventional mRNA capping mechanism in VSV

In the early 1970's, it was suggested that the cap structure of NNS viral mRNAs was formed by a mechanism which was unique from eukaryotic cap formation. For VSV [6], RSV [61], and spring viremia of carp virus [62], the two italicized phosphates of the 5′G*ppp*5′NpNpN triphosphate bridge have been shown to be derived from a GDP donor, rather than GMP. However, further studies on this mechanism have been seriously hampered due to the fact that the VSV capping events are tightly coupled to transcription and the capping machinery does not respond to exogenous transcripts. In 2007, this unique capping mechanism was revealed using a novel *trans* capping assay, in which a short mRNA corresponding to the first 5-nt of VSV gene start sequence was capped by a highly purified L protein in *trans* [12]. Specifically, capping of VSV mRNA was achieved by a novel polyribonucelotidyltransferase (PRNTase) which transferred a monophosphate RNA onto a GDP acceptor through a covalent L-RNA intermediate (Fig. 4C). In the first step, a GTPase associated with the VSV L protein removes the γ-phosphate group of GTP to generate GDP, an RNA acceptor. In the second step, the PRNTase activity of the L protein specifically transfers a 5′-monophosphorylated (p-) RNA moiety of pppRNA with the conserved VSV mRNA-start sequence (AACAG) to GDP to yield a GpppA capped mRNA. Interestingly, this

http://dx.doi.org/10.5772/54598

unusual VSV capping enzyme caps RNA in a sequence specific manner [12]. Specifically, VSV L protein efficiently capped pppApApCpApG (the mRNA gene start sequence), but not pppApCpGpApA (the leader RNA start sequence). In addition, the VSV L protein was not able to cap ppApApCpApG, suggesting that the L protein specifically recognizes the 5′-triphosphorylated AACAG. Further mutagenesis analysis has shown that the APuCNG (Pu, purine) sequence acts as a *cis*-acting element for the RNA capping reaction for the VSV L protein [12].

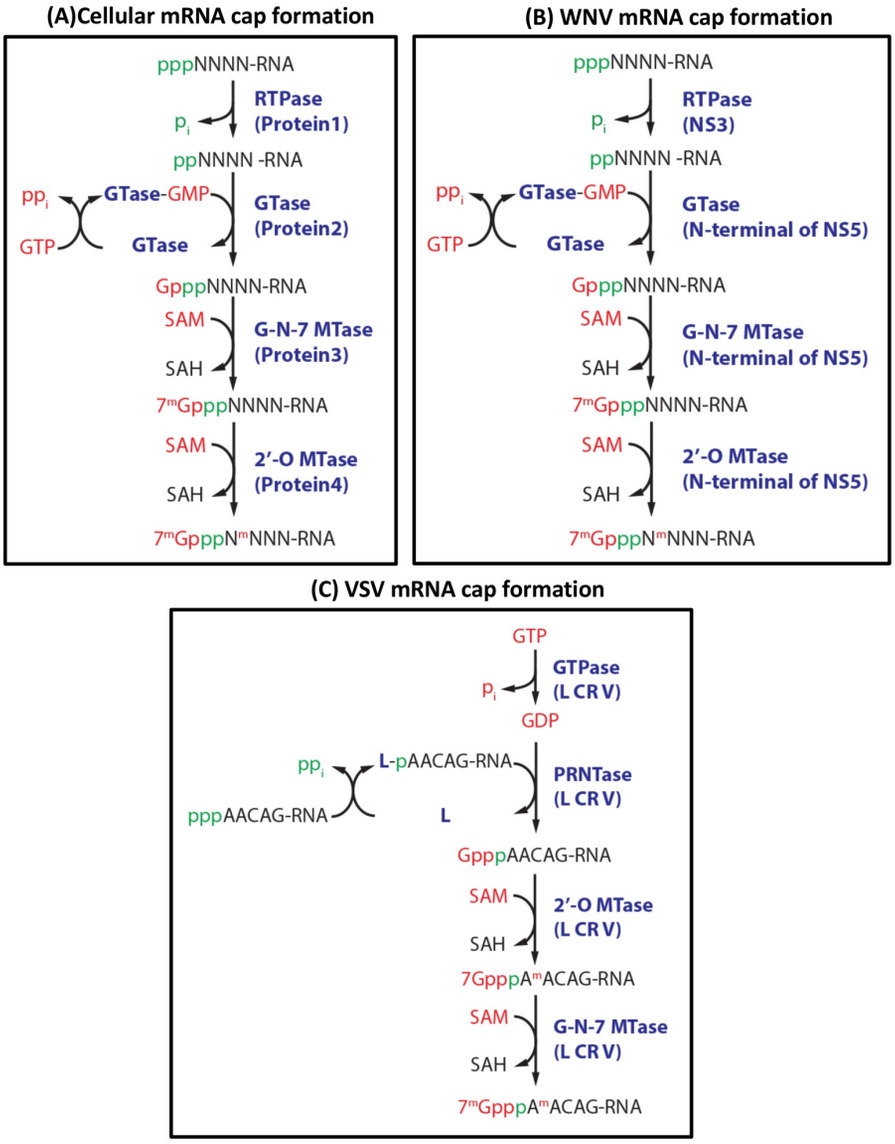

Figure 4. Comparison of mRNA cap formation in eukaryotic cells, WNV, and VSV. (A) Cellular mRNA cap formation. First, pppNNNN-RNA is hydrolyzed by an RNA triphosphatase (RTPase) to yield the diphosphate ppNNNN-RNA. Second, an RNA guanylyltransferase (GTase) transfers GMP to ppNNNN-RNA to yield GpppNNNN-RNA. Third, GpppNNNN-RNA is methylated by G-N-7 MTase to yield 7^{m}GpppNNNN-RNA. Fourth, 7^{m}GpppN-RNA is further methylated by a 2′-O MTase to yield 7^{m}GpppN^{m}NNN-RNA. **(B) WNV mRNA cap formation.** First, pppNNNN-RNA is hydrolyzed by viral NS3 protein (RTPase) to yield ppNNNN-RNA. Second, viral NS5 protein (GTase) transfers GMP to ppNNNN-RNA to yield GpppNNNN-RNA. Third, GpppNNNN-RNA is methylated by N-terminus of NS5 (G-N-7 MTase) to yield 7^{m}GpppNNNN-RNA. Fourth, 7^{m}GpppN-RNA is further methylated by N-terminus of NS5 (2′-O MTase) to yield

$7^{m}GpppN^{m}NNN$-RNA. **(C) VSV mRNA cap formation.** First, a GTPase (CR V of L protein) removes the γ-phosphate group of GTP to generate GDP. Second, a polyribonucelotidyltransferase (PRNTase) (CR V of L protein) transfers a monophosphate RNA onto a GDP acceptor through a covalent L-RNA intermediate to GpppAACAG-RNA. Third, the cap structure is methylated a 2′-O MTase (CR VI of L protein) to yield $GpppA^{m}ACAG$-RNA. Fourth, $GpppA^{m}ACAG$-RNA is further methylated a G-N-7 MTase (CR VI of L protein) to yield $7^{m}GpppA^{m}ACAG$-RNA.

It has been a challenge to locate the active site for the novel PRNTase in the 241 kDa L protein. The only suggestive information regarding the location of the capping enzyme in L protein has come from the study of a novel inhibitor of the RSV polymerase which resulted in the synthesis of short uncapped viral RNAs *in vitro* [63]. Viral mutants resistant to this inhibitor were selected, and the resistance mutations were mapped to CR V, suggesting that CR V of L plays a role in mRNA cap formation. Sequence alignments of this region of L protein identified a total of 17 residues that were conserved among the NNS RNA viruses [16]. Guided by this information, an extensive mutagenesis analysis was performed within this region which led to the discovery of a new motif GxxT[n]HR composed of four amino acid residues (G1154A, T1157A, H1227A, and R1228A) in VSV L protein which are essential for mRNA cap formation [16]. *In vitro* RNA reconstitution assays have shown that these cap defective polymerases synthesized uncapped mRNAs that terminated prematurely. The size of these abortive transcripts ranged from 100 nt up to the full-length N mRNA, although the majority were less than 400 nt. Consistent with their inability to generate capped RNA during *in vitro* transcription reactions, G1154A, T1157A, H1227A, and R1228A were defective in *trans* capping of the 5-nt VSV gene start sequence, demonstrating that these amino acids in CR V of L protein are required for mRNA cap addition [16]. Importantly, GxxT[n]HR is highly conserved in the CR V of L proteins of all NNS RNA viruses, including Borna disease virus which replicates in nucleus. Further biochemical and mass spectrometric analyses found that H1227 in the conserved GxxT[n]HR motif of the VSV L protein is covalently linked to the 5′-monophosphate end of the RNA through a phosphoamide bond [13]. Therefore, amino acid residue H1227 is the active site of the PRNTase activity. Mutagenesis analysis also found that R1228A and R1228K mutations significantly decreased L-pRNA complex formation activities, suggesting that mutation in R1228 may affect the H1227-RNA intermediate formation [13]. Interestingly, this PRNTase activity was also found in L protein of Chandipura virus (CHPV), a rhabdovirus that is closely related to VSV [64]. Furthermore, mutations to HR motif in L protein of CHPV significantly reduced the formation of the L-pRNA covalent intermediates in the PRNTase reaction. These results demonstrate that this unconventional capping mechanism is conserved in the *Rhabdoviridae* family. Given the fact the HR motif is highly conserved in L proteins of NNS RNA viruses, it is likely that this novel capping mechanism is not only unique to rhadoviruses, but also may be utilized by other NNS RNA viruses.

5. An unusual mechanism of mRNA cap methylation in VSV

5.1. Conventional mRNA cap methylation in eukaryotic cells

In eukaryotic cells, the capped mRNA (GpppN-RNA) is typically methylated by two steps (Fig.4A) [65-68]. First, the capping guanylate is methylated by a G-N-7 methyltransferase

http://dx.doi.org/10.5772/54598

(MTase) to yield 7^{m}GpppN-RNA (cap 0). Second, the G-N-7 methylated cap structure can then be further methylated by a ribose-2′-O (2′-O) MTase to yield 7^{m}GpppN^{m}-RNA (cap 1). During mRNA cap methylation, S-adenosyl-L-methionine (SAM) serves as the methyl donor, and the by-product S-adenosyl-homocysteine (SAH) is the competitive inhibitor of the SAM-dependent MTase. These mRNA cap methylation reactions are conserved among all eukaryotes. In this conventional methylation reaction, G-N-7 methylation occurs prior to 2′-O methylation and the two methylase activities are carried out by two separate enzymes, each containing its own binding site for the methyl donor, SAM.

Many viruses encode their own mRNA cap methylaion machinery, the best-studied example of which is the poxvirus vaccinia virus. For vaccinia virus, the G-N-7 and 2′-O MTase activities are encoded by two separate viral proteins, D12L and VP39 [65, 68-70]. In the case of reovirus, G-N-7 and 2′-O MTases are catalyzed by two separate domains of the same viral polymerase protein [55, 71]. For VSV, G-N-7 and 2′-O MTases are accomplished by a single region (CR VI) located in the C terminus of viral polymerase protein, L (Fig.4C) [14, 17, 19]. Soon after the discovery of the dual MTase activities of VSV, the N terminus of flaviviruses polymerase protein (NS5) was found to encode both G-N-7 and 2′-O MTases (Fig.4B) [72-74]. In addition to this unusual dual MTase activity of CR VI, the order of mRNA cap methylation in VSV is unconventional in which 2′-O methylation precedes and facilitates the G-N-7 methylation [17, 18]. This is contrast to all known mRNA cap methylation reactions including flaviviruses.

5.2. A single MTase catalytic site in CR-VI of L protein essential for both G-N-7 and 2′-O methylation

The SAM-dependent MTase superfamily contains a series of conserved motifs (X and I to VIII) [75]. The crystal structure of several known 2′-O MTases including *E. coli* heat shock-induced methyltransferase RrmJ/FtsJ and vaccinia virus VP39 have been solved[67, 68, 70, 76]. In RrmJ, a catalytic tetrad of residues: K38, D124, K164, and E199 formed the active site of 2′-O MTase [67, 76]. Site-directed mutagenesis of RrmJ found that a catalytic triad of residues K38, D124, and K164 are essential for 2′-O MTase whereas E199 plays only a minor role in the methyltransferase reaction *in vitro*. In vaccinia virus VP39, four amino acids, K41, D138, K175, and E207, are essential for catalysis [68, 70]. By comparing the amino acid sequence of the RrmJ and VP39 with CR VI of the L protein of NNS RNA viruses, it was suggested that this region of L protein might function as a 2′-O MTase. Sequence alignments suggest that residues K1651, D1762, K1795, and E1833 of the VSV L protein correspond to a catalytic KDKE tetrad (Fig.5). In fact, this KDKE motif is conserved in CR VI of L proteins of all NNS RNA viruses with the exception of Borna disease virus. Li et al., (2005) performed an extensive mutagenesis analysis in this predicted MTase catalytic KDKE tetrad in VSV L protein [14]. Recombinant VSVs carrying individual substitutions to K1651, D1762, K1795, and E1833 were recovered from an infectious cDNA clone of VSV. Analysis of the cap structure of mRNA synthesized *in vitro* revealed that alterations to the predicted active site residues abolished both G-N-7- and ribose 2′-O MTase activities. This result demonstrated that a single KDKE tetrad in CR-VI of the VSV L protein is essential for mRNA cap G-N-7- and

ribose 2′-O methylation [14]. Two models have been proposed to explain this result. One possibility is that CR VI functions as both G-N-7 and 2′-O MTases. However, this conflict the fact that all known G-N-7 and 2′-O MTases have distinct biochemistry during RNA methylation reactions. An alternative explanation is that there is a sequential model for VSV mRNA cap methylation in which the product of one MTase acts as the substrate for the second (discussed below).

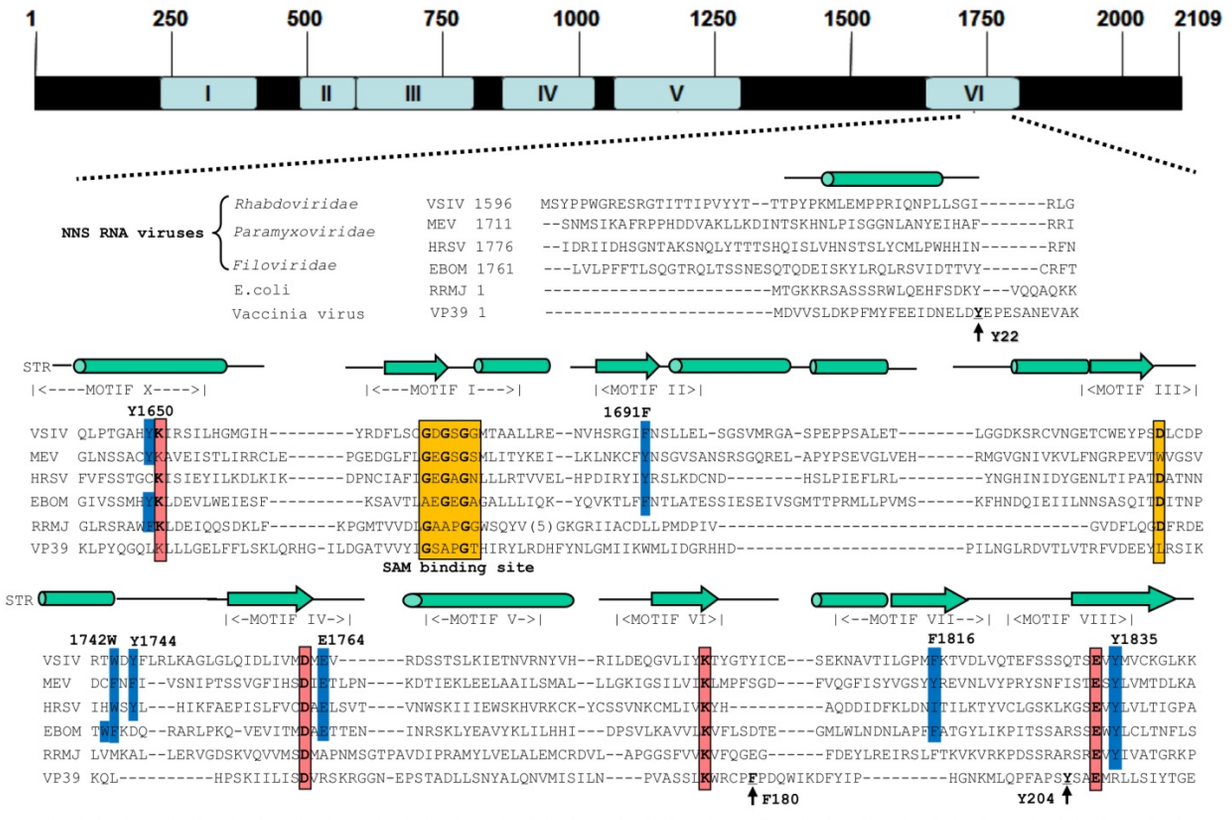

Figure 5. Structure-based amino acid sequence alignments of conserved domain VI of representative NNS RNA virus L proteins with known 2'-O methyltransferase, the *E. coli* RrmJ and vaccinia virus VP39. The conserved motifs (X and I to VIII) correspond to the SAM-dependent MTase superfamily. MTase catalytic site is shown by pink color. SAM binding site are shown by yellow color. Conserved aromatic residues are shown by blue color. Predicted alpha-helical regions are shown by the cylinders and the ß-sheet regions by the arrows. STR, structure of RrmJ and predicted structure for the NNS RNA viruses; EBOM, Ebola virus; VSIV, VSV Indiana type; HRSV, human respiratory syncytial virus; RRMJ, *E. coli* heat shock 2'-O MTase; VP39, vaccinia virus 2'-O MTase VP39.

http://dx.doi.org/10.5772/54598

5.3. A single SAM binding site in L protein essential for both G-N-7 and 2′-O methylation

The SAM-dependent MTase superfamily usually contains a G-rich motif (GxGxG) and an acidic residue (D/E) that is involved in SAM binding [75]. Sequence alignments between CR VI of NNS RNA virus L proteins and known MTases suggest that the SAM-binding residues of VSV L include G1670, G1672, G1674, G1675, and D1735 (Fig.5). Site-directed mutagenesis has been performed to define the roles of these amino acids in VSV mRNA methylation [17]. Each of these residues was individually substituted for alanine (A); or, for G4A, all four G residues were replaced with A; for G4AD, residue D1735 was also replaced with A. In addition, the flanking amino acid residues D1671 and S1673 within GDGSG motif were also substituted. Recombinant viruses were recovered from each of the *L* gene mutations. It was found that mutations to G1670, G1672, and S1673 specifically diminished G-N-7, but not 2′-O methylation, suggesting that 2′-O methylation occurs prior to G-N-7 methylation in VSV [17]. In contrast, mutants D1671, G4A, G4AD, G1675A, and D1735 were defective in both 2'-O and G-N-7 methylations. Interestingly, mutant G1674A requires a higher concentration of SAM to achieve full methylation compared with wild type VSV and methylation is more sensitive to SAH inhibition. Therefore, amino acid substitutions to the predicted SAM binding site disrupted methylation at the G-N-7 position or at both the G-N-7 and ribose 2′-O positions of the mRNA cap. However, none of these mutants are specifically defective in 2′-O methylation alone. These studies provide genetic evidence that the two methylase activities share one single SAM binding site and, in contrast to other cap methylation reactions, methylation of the G-N-7 position is not required for 2′-O methylation.

5.4. Mapping the potential RNA binding site that required for mRNA cap methylation

To acquire methylation, the MTase usually directly or indirectly contacts an RNA substrate. This putative substrate binding site is poorly understood in NNS RNA viruses. However, this substrate binding site has been identified in several cellular and viral mRNA 2'-O MTases [67, 68, 70, 74, 77, 78]. To achieve 2'-O methylation, the RNA substrate interacts with the cap recognition site which requires stacking between the base of the cap and aromatic rings from a MTase [76, 79, 80]. Vaccinia VP39 is one of the best characterized 2'-O MTases. In VP39, it was found that the recognition of a methylated base is achieved by stacking between two aromatic residues (Y22 and F180) and the methyl group is in contact with residue Y204 (Fig.6A) [68, 70, 79]. In addition, the carboxyl groups of residues D182 and E233 form hydrogen bonds with the NH and NH2 of the guanosine in VP39 (Fig.6A). Based on structure modeling and mutagenesis analysis, it was shown that residue F24 in West Nile virus (WNV) methylase (NS5) [81, 82] and Y29 and F173 in feline coronavirus 2'-O MTase (nsp16) [80] may play an equivalent role to residue Y22 in VP39 of vaccinia virus. The cellular cap binding protein-eukaryotic translation initiation factor 4E (eIF-4E) recognizes the cap by stacking between W56 and W102 [83]. In all known cases, aromatic residues are involved in cap binding and substrate recognition.

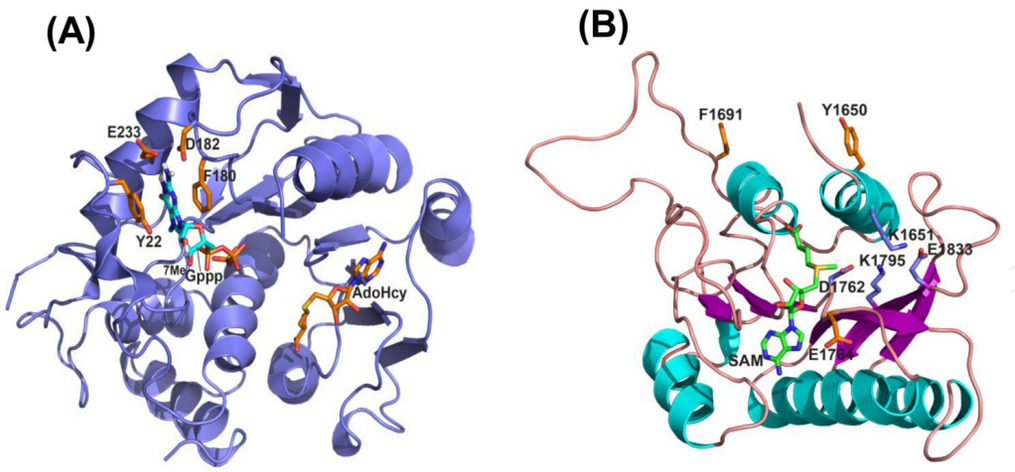

Figure 6. The predicted structure of CR VI of VSV L protein. **(A) Cap binding site of vaccinia virus 2'-O MTase, VP39**. The model was generated from the crystal structure of VP39 (PDB code: 1AV6) using PyMOL. The side chains of amino acid residues (Y22, F180, E233 and D182) involved in binding of 7^{m}Gp cap are shown as sticks. **(B) The predicted structure of CR VI of VSV L protein**. The model was generated based on the previous predicted structure of VSV MTase (amino acid residue from 1644 to 1842 in L protein) using PyMOL software. Alpha helices are shown in blue and beta strands are shown in pink. SAM and the side chains of critical residues are shown in sticks, and the oxygen atoms and nitrogen atoms are shown in red and blue, respectively. For SAM molecule, the carbon atoms are green. For the three important amino acids (Y1650, F1691 and E1764) that may be involved in RNA substrate binding, their side-chain carbon atoms are highlighted in orange. For the predicted catalytic residues (K1651, D1762, K1795 and E1833), the carbon atoms are shown in purple.

Guided by this information, the putative RNA binding site in VSV L protein was searched through mutagenesis analysis of selected conserved residues in region VI of VSV L protein that were physiochemically similar to those involved in substrate recognition in VP39. Sequence alignment showed that there are a number of aromatic residues that are highly conserved in the MTase domain of L proteins of NNS RNA viruses (Fig. 5). Aromatic residues at positions 1650 (Y), 1691(F or Y), and 1835 (Y) are highly conserved in L proteins of NNS RNA viruses. Aromatic residues at positions 1742 (W), 1744 (Y), 1745 (F), and 1816 (F) are conserved in the L proteins of *Rhabdoviridae* and some *Paramyxoviridae* and *Filoviridae*. Therefore, these aromatic residues were selected as putative equivalents of Y22, F180, and Y204 in VP39. However, there is no amino acid precisely aligned with D182 and E233 in VP39. With the exception of two acidic amino acids in the catalytic site (K1651-D1762-K1795-E1833), position E1764 is also conserved in all L proteins. Thus, E1764 was selected as a candidate for mimicking the role of VP39 residues D182 and E233. In addition, two serine mutations at the two most conserved positions at 1693 and 1827 of VSV L protein was also examined, based on the fact that it has been shown that a serine residue was involved in RNA-protein interaction in *E. coli* 2'-O MTase, RRMJ [67, 77]. To determine the role of these amino acid residues in mRNA cap methylation, a single point mutation was introduced to an infectious clone of VSV and recombinant VSVs harboring these mutations were recovered [84]. The importance of the maintenance of the aromatic ring at amino acids Y1650 and F1691 was revealed by the observation that the substitution of Y1650 and F1691 with two other possible aromatic residues in the VSV infectious clone still produced viable recombinant viruses and produced a fully methylated mRNA cap, but alanine substitutions dramatically inhibited viral replica-

http://dx.doi.org/10.5772/54598

tion and completely blocked both G-N-7 and 2′-O methylation [84]. Based on the predicted structural model for the VSV MTase (amino acid residues from 1644 to 1842 in the L protein) (Fig.6B), the residues Y1650 and F1691 are located far from each other with a distance of 17.3 Å between their alpha carbon atoms. Y1650 is located in the middle of the first helix, and the F1691 is at the very C-terminal of the second helix. Perhaps, a stacking interaction with one aromatic residue causes a conformational and structural change in the VSV methylase, which results in the interaction with another aromatic residue. Changing of residue E1764 to D (maintenance of charge), Q (maintenance of size), or K (changing charge), even the very conservative change to D, dramatically inhibited both G-N-7 and 2'-O methylation [84]. The predicted structure of VSV MTase also shows that E1764, the residue adjacent to the catalytic residue D1762, is exposed to the putative SAM binding site (Fig.6B). The side chain of E1764 shows close contact to the adenyl group of SAM (3.1 Å). In addition, it was found that Y1835A was found to require a higher SAM concentration to achieve full methylation and it is more sensitive to MTase inhibitor [84].

To date, this work is the first attempt toward elucidation of the putative RNA substrate recognition site in the L protein of NNS RNA viruses, which has shed light on the possible role of several conserved aromatic amino acids, including Y1650 and F1691, in RNA binding during cap methylation. It would provide much more direct evidence for the role of these key amino acids in mediating RNA binding if the RNA binding efficiency could be measured directly. Attempts to use a gel shift assay have failed to this end [84], as the existence of multiple RNA binding sites in L protein with a size as large as 241-kDa posed a tremendous challenge in discerning the effect of single point mutation. The use of a truncated CR VI of VSV L for in vitro RNA binding assays might be a useful alternative strategy for future studies.

5.5. An unusual order for mRNA cap methylation in VSV

For conventional mRNA cap methylation, two separate MTases sequentially methylated the cap structure, first at the G-N-7 position and subsequently at the ribose 2′-O position [65, 66]. Analysis of the cap methylation of mRNA synthesized *in vitro* suggests that mRNA cap methylation in VSV is unusual, with methylation of ribose 2′-O occurring prior to G-N-7 methylation. First, early studies showed that at low concentrations of SAM, VSV mRNA was methylated at the 2′-O position only [85]. However, it could be chased into a doubly methylated cap structure at high SAM concentrations *in vitro*. Second, when *in vitro* mRNA synthesis was performed in the presence of MTase inhibitors such as SAH and sinefungin, G-N-7 methylation was inhibited prior to 2′-O methylation [86]. Third, a host range mutant of VSV, *hr*8, was shown to synthesize mRNA cap structures that lacked G-N-7 but were partially 2′-O-methylated [46, 87]. Finally, VSV mutants carrying mutations in the SAM binding site (such as G1670A, and G1672A) are specifically defective in G-N-7, but not 2′-O methylation [17].

This unusual order of VSV mRNA cap methylation was also biochemically demonstrated by a *trans*-methylation assay in which both ribose 2′-O and G-N-7 MTases were recapitulated by using purified recombinant L and *in vitro*-synthesized RNA [18]. It was found that VSV L modifies the 2′-O position of the cap prior to the G-N-7 position and that G-N-7 methylation is diminished by pre-2′-O methylation of the substrate RNA [18], providing compelling evi-

dence that 2′-O methylation precedes and facilitates G-N-7 methylation. In light that both two MTase activities appear to reside in the same domain of L protein with the same SAM binding site, it is conceivable that mRNA cap and/or the SAM binding site might need to be repositioned at the end of the first methylation reaction to facilitate the second round of methylation. How this coordination happens *in vitro* is still a mystery. Bearing in mind that G-N-7 position is upstream of the ribose 2'-O position in the mRNA strand, reorientation is thus less likely to have resulted from forward movement of capped RNA through CR VI during transcription, but rather it might entail a fine spatial rearrangement. Collectively, these experiments have shown that the order of VSV mRNA cap methylation is distinct from all other known mRNA cap methylation mechanisms.

5.6. VSV methylases require *cis*-element in RNA

During mRNA synthesis, the VSV polymerase initiates synthesis at the first gene-start (GS) sequence (3′ UUGUCNNUAC 5′), and the nascent mRNA chain is capped and methylated, and recognizes a specific gene-end (GE) sequence (3′-AUACUUUUUUU-5′), the polymerase polyadenylates and terminates. It has been well demonstrated that the GS sequence contains a key *cis*-acting regulatory element for the initiation of mRNA synthesis [31, 32]. Specifically, the first three positions of the GS sequence have been found to be critical for mRNA synthesis. Recently, both *trans* capping assays with 5-nt oligo RNA substrates and detergent-activated virus transcription reactions pointed out the importance of positions 1, 2, 3, and 5 in mRNA cap addition, although position 5 substitutions were more tolerated [12, 31, 32]. Using a *trans* methylation assay, it was found that similar signals were required for mRNA cap methylation [18]. As expected, VSV L protein efficiently methylated a 110 nt of RNA with an authentic gene start sequence at position 2′-O. However, when the gene start sequence of this 110 nt was replaced with non-viral sequence (5′ GpppGGACGAAGAC-RNA), the efficiency of 2′-O methylation was reduced approximately 9 times. Similarly, VSV L protein efficiently methylated a pre-2′-O-methylated VSV mRNA at position G-N-7. In contrast, the efficiency of G-N-7 methylation decreased nearly 7 times when incubated with a substrate with non-VSV mRNA start RNA. Therefore, the gene start sequence of VSV mRNA contains a signal for initiation of mRNA synthesis, mRNA cap addition, and cap methylation.

5.7. The length of mRNA in cap methylation

In the *trans* mRNA capping assay, the VSV L protein efficiently caps the 5-nt gene start sequence [12, 16], demonstrating that a 5-nt RNA substrate is sufficient for mRNA cap addition. In order to determine the minimum length of RNA required for mRNA cap methylation, 5-,10-, 51-, and 110-nt RNAs were used as substrates for a *trans* methylation assay *in vitro* [18]. Interestingly, the 10-, 51-, and 110-nt RNAs were able to serve as substrates for both G-N-7 and ribose 2'-O methylations, whereas the 5-nt RNA was not methylated by the VSV L protein at either the G-N-7 or the ribose 2'-O position [18]. Therefore, in contrast to *trans* capping, a 5-nt substrate is not sufficient for trans methylation and likely the conserved positions 8, 9, and 10 in VSV gene start sequence are required for mRNA cap methylation. Clearly, the length of RNA required for methylation is longer than that required for capping by the VSV L protein.

http://dx.doi.org/10.5772/54598

5.8. Model for mRNA cap methylation

The process of VSV L protein-mediated cap methylation can be best summarized with the following model (Fig.7). Initially in response to a specific *cis*-acting element in the VSV gene start sequence, CR VI of L protein methylates the cap structure first at the 2'-O position to produce GpppA^{m}ACAG-RNA. The by-product of this reaction, SAH, is released from this reaction. Following 2′-O methylation, a second molecule of SAM binds to CR VI of L protein that may facilitate a subsequent methylation of the RNA at the G-N-7 position. Methylation at the 2'-O position favors G-N-7 methylation in the cap structure through a currently unknown mechanism. G-N-7 methlyation may be facilitated by the contact with the RNA molecule remains bound to the L protein at the end of the initial methylation at the 2'-O position. Another possibility is that CR VI of L protein exists in two different conformations. An initial conformation may favor binding of GpppRNA and SAM. Methylation of RNA at the 2'-O position perhaps induces a conformational change that facilitates the repositioning of the RNA for subsequent G-N-7 methylation, and/or favors the release of SAH as well as the binding of a subsequent molecule of SAM.

5.9. Comparison of mRNA cap methylation in VSV and WNV

To date, the rhabdovirus, VSV, and the flavivirus, WNV, are the two best characterized viruses that utilize a single region in the polymerase protein for both G-N-7 and 2'-O methylations. However, the mechanism of VSV mRNA methylation is distinct from that of the WNV system (Fig.4B and C). In VSV, 2'-O methylation precedes and facilitates subsequent G-N-7 methylation [17, 18]. However, WNV MTases modify the cap structure, first at the G-N-7 position and subsequently at the ribose 2'-O position [72, 73, 78]. In VSV, the G-N-7 and 2'-O MTases require similar conditions for methylation with an optimal pH at 7.0 [18]. In contrast, the G-N-7 and 2'-O MTases of WNV require an optimal pH at 6.5 and 10, respectively [72]. Both VSV and WNV MTases modify the RNA in a sequence-specific manner, but require different elements in the RNA substrate. VSV G-N-7 and 2'-O MTases require specific gene start sequences with a minimum mRNA length of 10 nucleotides [18]. In the WNV model, N-7 cap methylation requires the presence of specific nucleotides at the second and third positions and a 5' stem-loop structure within the 74-nucleotide viral RNA; in contrast, 2'-O ribose methylation requires specific nucleotides at the first and second positions, with a minimum 5' viral RNA of 20 nucleotides in length [81]. In addition, there is striking difference in the cap recognition site between the VSV and WNV MTases. For the WNV MTase, the cap recognition site is essential for 2'-O, but not G-N-7 methylation [73, 82]. Consistent with this finding, it was found that GTP and cap analogs specifically inhibited 2'-O, but not G-N-7 methylation [73, 82]. However, mutations to the putative RNA binding site in VSV L protein affected both G-N-7 and 2′-O methylations. GTP and cap analogs did not affect VSV mRNA cap methylation *in vitro*. Overall, the mechanism of VSV mRNA cap methylation is significantly different from that of WNV. Most recently, it was found that capping of flavivirus RNA is catalyzed by conventional RNA guanylyltransferase via a covalent GMP-enzyme intermediate [88]. However, VSV capping is catalyzed by a novel PRNTase [12, 13, 16, 89]. These studies suggest that VSV and perhaps other NNS RNA viruses have evolved a unique mechanism to add the cap to their mRNA and to methylate the cap structure.

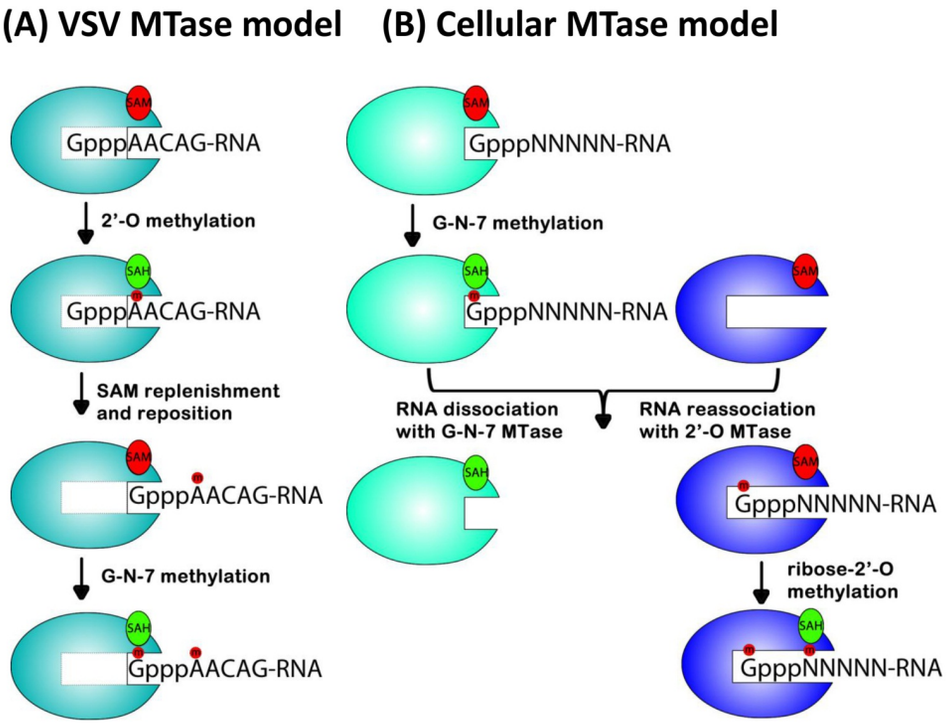

Figure 7. Proposed model for mRNA cap methylation in VSV and eukaryotic cells. (A) VSV MTase model. VSV mRNA cap structure is first methylated by CR VI of L protein at the 2'-O position to produce GpppA^{m}ACAG-RNA. The by-product, SAH, is released before the binding of a second molecule of SAM. G-N-7 methlyation may be facilitated by the contact with the RNA molecule remains bound to the polymerase at the end of the initial methylation at the 2'-O position. Or, methylation of RNA at the 2'-O position may induce a conformational change that facilitates the repositioning of the RNA for subsequent G-N-7 methylation. **(B) Cellular MTase model.** GpppNNNN-RNA is first methylated by a G-N-7 MTase to yield 7^{m}GpppNNNN-RNA. Following G-N-7 methylation, 7^{m}GpppNNNN-RNA dissociates with G-N-7 MTase, and re-associates with a separate 2'-O MTase to yield 7^{m}GpppN^{m}NNN-RNA. In this model, the two methylase activities are carried out by two separate enzymes, each containing its own SAM binding site.

5.10. mRNA cap methylation in other NNS RNA viruses

Limited accomplishments have been made in understanding the mechanism of mRNA cap methylation in other NNS RNA viruses, due to the lack of a robust *in vitro* mRNA synthesis for most of NNS RNA viruses, and the technical challenge of expression and purification of a functional polymerase protein or fragment. In early 1970, Colonno and Stone showed that the NDV mRNA cap structure was methylated only at G-N-7 [90]. This is distinct from the cap structures of other NNS RNA viruses which typically contain two methyl groups, at positions G-N-7 and ribose 2'-O. However, detailed characterization of the NDV methylase activities and the mechanism involved in this unique methylation is not understood. The mechanism underlying this difference and the biological significance of the lack of 2'-O MTase is not known. Sequence analysis has revealed that the proposed SAM binding region for the *Filoviridae*, *Rubulavirus*, and *Avulavirus* genera of the *Paramyxoviridae* contains a conserved AxGxG sequence rather than GxGxG within motif I of the SAM-dependent MTase superfamily (Fig.8). It will be interesting to determine if there is a link between this differential SAM binding sites and the lack of 2'-O methylation in NDV. Recently, it was shown that a fragment of Sendai virus L protein that includes CR VI was able to methylate short Sendai virus-specific RNA sequences *in trans* at the

http://dx.doi.org/10.5772/54598

G-N-7 position [43]. However, the "*trans*-methylation" assay used in their study does not allow detection of 2'-O methylation although it is known that Sendai virus encodes two MTases. More recently, recombinant Sendai virus carrying mutations in catalytic KDKE tetrad and SAM binding site were recovered from infectious clones [91, 92]. It was found that these mutations affected mRNA cap methylation. However, whether they are specifically defective in G-N-7 and/or 2'-O methylation is not known because of the assay did not have the ability to distinguish the two methyl groups. In addition, the order of methylation may be different among NNS RNA viruses. In contrast to VSV, methylation of RSV mRNA at low SAM concentrations was found at only the G-N-7 position; however, it was found to be doubly methylated at high SAM concentrations [63]. This study provided evidence that the order of mRNA cap methylation in RSV may be different with VSV. Clearly, more studies are needed to understand the mRNA cap methylation in other NNS RNA viruses.

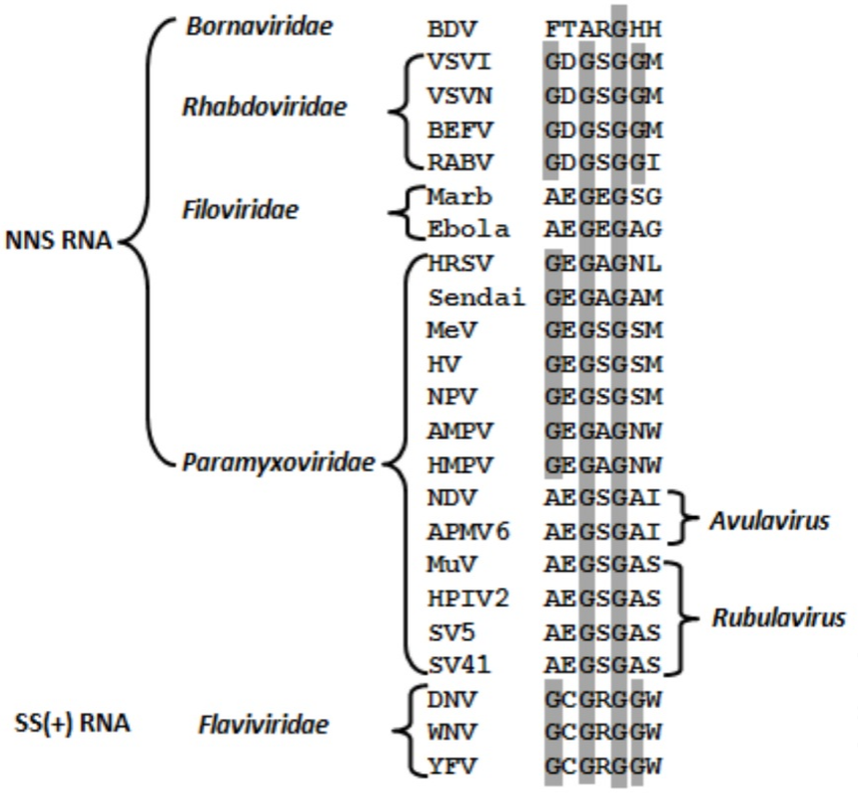

Figure 8. SAM binding motifs in NNS RNA virus MTases. The proposed SAM binding region for the *Filoviridae*, *Rubulavirus*, and *Avulavirus* genera of the *Paramyxoviridae* contains a conserved AxGxG sequence rather than GxGxG. NNS RNA viruses include VSVI, VSV Indiana; VSVN, VSV New Jersey; BEFV, bovine emphemeral fever virus; RABV, rabies virus; Marb, Marburg virus; MeV: measles; HV, Hendra virus; NPV, Nipah virus; AMPV, avian metapneuomovirus; HMPV, human metapneuomovirus; APMV6, avian paramyxovirus 6; MuV, mumps virus; HPIV2, human parainfluenza virus 2; SV5, simian virus 5; SV41, simian virus 41;

SS(+) RNA: single strand positive RNA viruses include DNV, dengue virus; WNV, West Nile virus; YFV, yellow fever virus.

6. The effects of 5′ mRNA cap addition and cap methylation on 3′ mRNA polyadenylation

VSV mRNA is capped and methylated at the 5′ end and polyadenylated at the 3′ end. Cap addition, cap methylation, and polyadenylation are carried out by three different regions (CR V, CR VI, and CR III) in L protein. During VSV mRNA synthesis, modifications of the 5′ and 3′ ends of the mRNAs are tightly coupled to transcription [21-23]. Although the detailed mechanism by which the polymerase coordinates these modification events is poorly understand, available evidence suggests there is a link between authentic 5′-end formation and 3′-end formation during VSV mRNA synthesis. Early studies demonstrated that the length of poly (A) tails on VSV mRNAs is affected by the presence of SAH, the by-product and competitive inhibitor of SAM-mediated methyltransferases [93-95]. The fact that the polymerase can synthesize full-length mRNAs *in vitro* in the absence of SAM or in the presence of SAH, suggests that transcription is not dependent on cap methylation. When *in vitro* transcription reactions are performed in the presence of SAM, the RdRp synthesizes mRNA with poly (A) tail of 100 to 200 nt in length, similar to those synthesized in VSV-infected cells. However, when VSV mRNA cap methylation was inhibited during *in vitro* transcription reactions by supplementing with 1 mM SAH, the synthesized mRNA was heterogeneous in length due to having extremely long poly (A) tails, from 700 to 2,400 nucleotides (nt) in length [93, 96]. Recent work demonstrated that SAH-induced hyperpolyadenylation also occurs in cells infected by wild-type VSV in the presence of adenosine dialdehyde (AdOX), a compound that inhibits the activity of SAH hydrolase [97]. These results indicated that chemical inhibition of VSV mRNA cap methylation by SAH resulted in hyperpolyadenylation of viral mRNA. Interestingly, this hyperpolyadenylation of VSV mRNAs has been observed in *ts*(G)16, a VSV mutant identified in 1970 based on its ability to grow at 31°C but not at 39°C [93, 98, 99]. In the absence of SAH, the mRNAs synthesized by VSV mutant *ts*(G)16 were hyperpolyadenylated at the 3′ end. Genomic sequence analysis found that the L protein of *ts*(G)16 contains two amino acid changes, C1291Y and F1488S, compared to wild type. Combined with the analysis of revertants of *ts*(G)16, it was found that F1488S, located in the variable region of L between CR V and CR VI, is responsible for the hyperpolyadenylating phenotype.

The characterization of a panel of MTase-defective VSVs may serve as a tool to understand the mechanism by which SAH or the failure to methylate the cap structure results in hyperpolyadenylation. It was found that rVSV-K1651A, a mutation in MTase active site and completely defective in G-N-7 and 2′-O methylation, synthesized excessively long poly(A) tails, similar to those produced by wild-type L in the presence of SAH [15]. Similarly, the substitution D1762E at the MTase active site, which inhibits both G-N-7 and 2′-O methylation, produces large polyadenylate in the presence or absence of SAH [97]. This data confirms the earlier work demonstrating that the inhibition of cap methylation results in large polyadenylate. In contrast, several other substitutions that inhibit cap methylation, including D1762G, D1762N, G1672P, and G1675P, did not produce hyperpolyadenylated mRNA [97].

http://dx.doi.org/10.5772/54598

Perhaps, K 1651A and D1762E substitutions might favor the binding of SAH at the SAM binding site in CR VI, resulting in hyperpolyadenylation without the need for supplemental SAH. Clearly, further studies are needed to understand the relationship between 5′ mRNA cap methyaltion and 3′ polyadenylation.

However, it appears clear that 5′ cap addition is required for 3′ polyadenylation, as evidenced by the polymerase mutants (G1154, T1157, H1227, and R1228) within CR V of L that inhibited cap addition also inhibit polyadenylation [15]. These cap-defective polymerases synthesized truncated transcripts that predominantly terminated within the first 500 nt of the N gene and contained short A-rich sequences at their 3′ termini. To examine how the cap-defective polymerases respond to an authentic VSV termination and re-initiation signal present at each gene junction, a 382 nt gene was inserted at the leader-*N* gene junction in the VSV genome. Using this N-RNA as the template, the cap-defective polymerases were able to synthesize full-length 382-nt transcripts that were not capped at 5′end. Interestingly, these uncapped transcripts lacked an authentic polyadenylate tail and instead contained 0 to 24 A residues [15]. In addition, the cap-defective polymerases were also unable to efficiently copy the downstream genes [15]. This finding strongly supports that 5′ mRNA cap addition and 3′ polyadenylation are mechanistically and functionally linked.

7. Impact of mRNA cap methylation on viral replication and gene expression

In eukaryotic cells, it is well established that G-N-7 methylation of the mRNA cap structure is essential for mRNA stability and efficient translation [11, 48, 66]. Specifically, G-N-7 methylation of the mRNA cap structure is required for recognition of the cap by the rate limiting factor for translation initiation, eIF-4E [100, 101]. The mRNA cap structures of NNS RNA viruses are typically G-N-7 and 2′-O methylated. Although the precise mechanism by which VSV mRNAs are translated is unclear, they are broadly thought to utilize a variation of the canonical cap-dependent translational pathway [102-104]. In VSV-infected cells, host mRNA translation is rapidly inhibited through the suppression of the intracellular pools of eIF-4E by a manipulation of the phosphorylation status of the 4E binding protein (4E-BP1) [104]. Nevertheless, *in vitro* experiments have shown that G-N-7 cap methylation facilitates translation of VSV proteins. MTase-defective VSV would provide a tool to study the role of mRNA cap methylation in viral protein synthesis. Ultimately, it will affect viral genome replication and gene expression since viral replication requires ongoing protein synthesis.

Based on the status of mRNA methylation, MTase-defective VSVs can be classified into three groups [14, 17, 84]. Viruses in the first group are completely defective in both G-N-7 and 2′-O methylation, including mutations in MTase active site (rVSV-K1651A, D1762A, K1795A, E1833Q, and E1833A), SAM binding site (rVSV-D1671V, G1675A, G4A, and G4AD), and putative RNA binding site (rVSV-Y1650A, F1691A, and E1764A). Viruses in the second group are specifically defective in G-N-7, but not 2′-O MTase, including mutants in SAM binding site (rVSV-G1670A, G1672A, and S1673A). Viruses in third group that require

elevated SAM concentrations to permit full methylation including a mutant in SAM binding site (rVSV-G1674A) and putative RNA binding site (rVSV-Y1835A). With the exception of rVSV-G1674A and Y1835A, all MTase-defective VSVs were attenuated in cell culture as judged by diminished viral plaque size, reduced infectious viral progeny release (in single-step growth curves), and decreased levels of viral genomic RNA, mRNA, and protein synthesis. It appears that the degree of attenuation is consistent with the defects of the methylation. For example, viruses defective in both G-N-7 and 2′-O methylation had 2-5 log reductions in growth whereas viruses only defective in G-N-7 had 1-2 log declines in replication [14]. Recombinant rVSV-G1674A and Y1835A replicated as efficiently as wild type rVSV [14]. A remarkable finding is that some of the mutants in the SAM binding site (rVSV-G1675A, G4A, and G4AD) affected transcription and replication differently [17]. For these mutants, replication was enhanced 2.5- to 4-fold, and transcription decreased up to 8-fold compared with rVSV. One feature of the gene expression strategy of NNS RNA viruses is that the polymerase complex controls two distinct RNA synthetic events: genomic RNA replication and mRNA transcription [20, 105, 106]. It is possible that SAM binding influences the switch of polymerase between replicase and transcriptase. Perhaps, L protein with SAM binding favors to function as transcriptase, whereas L protein that lacks SAM binding favors replicase function.

8. Impact of mRNA cap methylation on viral pathogenesis *in vitro*

Although it is well studied that MTase-defective viruses were attenuated in cell culture, the impact of mRNA cap methylation on viral pathogenesis *in vitro* is poorly understood. Recently, Ma et al., (2012) examined the pathogenicity of MTase-defective VSVs in mice [107]. VSV infects a wide range of wild and domestic animals such as cattle, horses, deer, and pigs, characterized by vesicular lesions in the mouth, tongue, lips, gums, teats, and feet. Although the mouse is not the natural host of VSV, it represents an excellent small animal model to understand VSV pathogenesis because VSV causes systemic infection and fatal encephalitis [108-110]. After intranasal inoculation, VSV infects olfactory neurons in the nasal mucosa and subsequently enters the central nervous system (CNS) through the olfactory nerves. The virus is then disseminated to other areas in the brain through retrograde and possibly anterograde trans-neuronal transport, ultimately causing an acute brain infection. It was found that VSV mutants, rVSV-K1651A, D1762A, and E1833Q, which have mutations in the MTase catalytic site and are defective in both G-N-7 and 2′O methylation, were highly attenuated in mice [107]. Mice inoculated with these recombinant viruses did not show any clinical signs of VSV infection such as weight loss, ruffled fur, hyperexcitability, tremors, circling, and paralysis. Furthermore, these mutant viruses were not able to enter the brain, had dramatic defects in replication in lungs, and did not cause significant histopathological changes in lungs and brain. Recombinant rVSV-G1670A and G1672A, which have mutations in the SAM binding site and are defective in G-N-7 but not 2′-O methylation, retained low virulence in mice [107]. Mice inoculated these two recombinants exhibited weight loss of approximately 2-3 g during days 3-7 post-inoculation and showed mild illnesses such as ruffled coat for 2-3

http://dx.doi.org/10.5772/54598

days but recovered quickly. But, none of mice in rVSV-G1670A and G1672A had neurological symptoms. These two recombinants had moderate defects in replication in lungs and brain, and caused moderate histopathological changes in lungs. Interestingly, recombinant rVSV-G4A, which carries four mutations (G1670A, G1672A, G1674A, and G1675A) in SAM binding site and is completely defective in both G-N-7 and 2′-O methylation, exhibited an interesting pathotype [107]. Similar to VSV mutants at the MTase catalytic site, rVSV-G4A had dramatically impaired viral replication in the lungs and brain of the mice. However, the rVSV-G4A group exhibited body weight losses that were comparable to G1670A and G1672A, and clinical signs that were more severe than G1670A and G1672A. These results suggest rVSV-G4A retained low to moderate virulence despite the fact that it was attenuated for replication and or spread *in vitro*. As predicted, recombinant rVSV-G1674A, which contains a point mutation in the SAM binding site and requires elevated SAM concentrations to permit full methylation, was highly virulent to mice. These results suggest that (i) the relationship between mRNA cap methylation and viral pathogenesis is not clear cut in VSV infections; and (ii) inactivation of the predicted catalytic residues attenuates the virus to a greater extent *in vitro*, than does inactivation of the predicted SAM binding site.

9. 2′-O methylation and innate immunity

While it is firmly established that G-N-7 methylation is essential for mRNA stability as well as efficient translation, the role(s) of ribose 2′-O methylation have proven more elusive. Recent studies on West Nile virus (WNV) suggest that the 2′-O methylation of the 5′ cap of viral RNA functions to evade innate host antiviral responses through escape of the suppression of interferon-stimulated genes, tetratricopeptide repeats (IFIT)[111]. Specifically, mutant WNV (E218A) defective in 2′-O MTase activity was attenuated in wild-type C57BL/6 mice, but remained pathogenic in knockout mice that lacked the type I interferon (IFN) signaling pathway. In addition, a vaccinia virus mutant (J3-K175R) and mouse hepatitis virus (MHV) mutant D130A, both of which lacked 2′-O MTase activity, exhibited enhanced sensitivities to the antiviral actions of IFN mediated by IFIT proteins. Interestingly, it was also reported that 2′-O methylation of mouse and human coronavirus RNA facilitates evasion from detection by the cytoplasmic RNA sensor Mda5 [112]. Taken together, these studies suggest that 2′-O methylation of viral RNA provides a molecular signature for the discrimination of self and non-self mRNA. It is known that mRNAs of most NNS RNA viruses contain G-N-7 and 2′-O methylation. However, whether 2′-O methylation plays a similar role in all NNS RNA viruses is not known.

VSV is an excellent model to aid in the understanding the role of viral mRNA cap methylation in innate immunity. The mechanism of VSV mRNA cap methylation is unique in that 2′-O methylation precedes and facilitates the G-N-7 methylation [17, 18]. It is unknown why the order of VSV mRNA methylation is reversed compared to all known mRNA cap methylation reactions. One possibility is that the methylation of 2′-O allows VSV to successfully mimic cellular mRNA to avoid the detection by host innate immunity, which in turn promotes efficient viral replication in hosts. In fact, VSV is one of only a few viruses that repli-

cates efficiently in a wide range of cell lines including mammalian cells, insect cells, and worms [113]. Unlike WNV, VSV that is specifically defective in G-N-7 methylation can also be successfully recovered [17]. VSV mutants defective in G-N-7 methylation or both G-N-7 and 2′-O methylations can serve as prototypes or controls to elucidate the role of methylation in innate immunity. In addition, the VSV mutant (rVSV-G4A) that was attenuated in cell culture retained low to moderate virulence, suggesting the possible role of methylation in averting the innate immune response [107]. Notably, mRNAs of NDV, an avian paramyxovirus, are not 2′-O methylated [90]. A direct comparison to determine whether 2′-O methylation of VSV has a similar biological function compared to WNV and MHV should prove very compelling.

10. MTase-defective viruses as live vaccine candidates

Recombinant viruses defective in MTase can be recovered from cloned full-length viral cDNA by a reverse genetics system. Viruses lacking MTase would likely be attenuated without affecting immunogenicity, since the MTase is located in L protein, which is not a neutralizing antibody target. Our group and others have identified a panel of MTase-defective VSV mutants which are attenuated in cell culture as well as in animal models [14, 17, 84, 107]. In addition, MTase-defective Sendai viruses also showed significant defects in viral growth in cell culture [91, 92]. By combining multiple substitutions within the methylation region, it should be possible to generate an attenuated virus that is genetically stable, as reversion to wild type at any single amino acid should not provide a fitness gain. Thus, ablating viral mRNA cap methylation would provide a new avenue to rationally attenuate these viruses for the development of live attenuated vaccines and exploit their use as viral vectors for vaccines, oncolytic therapy, and gene delivery. Recently, Ma et al., (2012) showed that MTase-defective VSVs were able to induce high levels of VSV-specific antibodies in mice and thus provided full protection against a virulent challenge with the VSV Indiana serotype [107]. Recombinants rVSV-K1651A, D1762A, and E1833Q which were defective in both G-N-7 and 2′-O methylation, are attractive vaccine candidates since they are not only highly attenuated but also retain high immunogenicity. Although recombinants rVSV-G1670A and G1672A retained low virulence to mice, their pathogenicity was significantly reduced compared to rVSV. The safety of using these two viruses as live vaccine candidates necessitates further investigation.

Our studies on MTase-defective VSVs also shed light on developing live vaccine candidates for other NNS RNA viruses, particularly paramyxoviruses. Within paramyxoviruses,

RSV, hMPV, and PIV3 account for the majority of respiratory diseases infants, children, and the elderly [1-3]. However, there is no vaccine available for these important viruses. Recent research found that live attenuated vaccines are the most promising vaccine candidates for paramyxoviruses [1-3]. However, it has been technically challenging to isolate a virus with low virulence while retaining high immunogenicity. Introducing mutations in the MTase may provide a novel approach to generate live attenuated viruses for these

http://dx.doi.org/10.5772/54598

viruses. It was reported that recombinant Sendai virus carrying point mutations in the MTase catalytic site (rSeV-K1782A) and the SAM binding site (rSeV-E1805A) were attenuated in cell culture [91]. It will be of interesting to determine whether these Sendai recombinants are attenuated *in vitro*.

11. mRNA cap methylation as a target for anti-viral drug discovery

It appears that the entire mRNA capping and methylation machinery in NNS RNA viruses is different from that of their hosts. This difference, coupled with the fact that replication of NNS RNA viruses occurs in the cytoplasm, suggests that mRNA cap formation is an excellent target for anti-viral drug discovery. Inhibition of the viral mRNA cap formation would likely inhibit downstream events such as replication, gene expression, viral spread, and ultimately viral infection. Since the mRNA of all NNS RNA viruses contains a methylated cap structure, classes of broadly active anti-viral agents may be developed by targeting the viral cap formation. For human RSV, several compounds were shown to inhibit polymerase activity which resulted in the synthesis of short uncapped transcripts [63]. RSV mutants resistant to these inhibitors were selected and sequenced. It was found that these resistant mutants contained substitutions in CR V of L, specifically at E1269D, I1381S, and L1421F, suggesting that the mechanism of the action of these compounds is the inhibition of viral mRNA cap addition. Interestingly, these compounds showed strong antiviral activity against RSV infection in cell culture as well as in a mouse model, demonstrating that mRNA cap addition is an attractive antiviral target. It is known that SAH can inhibit viral mRNA cap methylation. Therefore, many adenosine analogues such as 3-deazaeplanocin-A are potent antiviral agents which can significantly inhibit VSV replication in cell culture [114, 115]. The mechanism of the action of these adenosine analogues is through the interference with the host enzyme SAH hydrolase that catalyzes the hydrolysis of SAH to adenosine and L-homocysteine. This reaction is reversible, and the products of this reaction are inhibitory to SAH hydrolase. Obviously, compounds that directly inhibit viral mRNA cap methylation are potent antiviral drugs. For example, sinefungin (SIN), a natural *S*-adenosyl-l-methionine analog produced by *Streptomyces griseolus*, is an inhibitor of methyltransferases. SIN is structurally related to SAM, with the exception that the methyl group that is donated from SAM is replaced by an amino group in SIN. Crystal structures of several MTases have been solved in complex with SIN, which binds to a region that overlaps the SAM binding site [116]. It was found that SIN inhibited VSV G-N-7 and 2′-O methylation with a 50% inhibitory concentration (IC_{50}) of 2.5 μM and 40 μM, respectively [86]. In cell culture, SIN efficiently inhibited VSV replication, gene expression, and diminished the size of viral plaques without having significant effect on cell viability. SIN was also shown to inhibit the MTases of other NNS RNA viruses such as NDV [90]. These examples demonstrate that mRNA capping and methylation is an excellent antiviral target for NNS RNA viruses. An important future direction is to develop high-throughput screening (HTS) to systemically screen compounds that can inhibit mRNA capping and/or methylation of VSV and other NNS RNA viruses. These inhibitors may be broadly active anti-viral agents against NNS RNA viruses.

12. Concluding remarks

In recent years, significant progress has been made in understanding the unusual mechanism of mRNA cap addition and methylation employed by VSV. First, VSV mRNA addition utilizes a novel PRNTase that transfers RNA to the GDP acceptor. Second, VSV mRNA cap methylation is catalyzed by a dual MTase that sequentially methylates the position 2'-O followed by G-N-7. Third, PRNTase and dual MTase have been mapped to single amino acid level in CR V and CR VI in the L protein, respectively. Finally, 5' mRNA cap addition and methylation and 3' polyadenylation are mechanistically and functionally linked. Apparently, the entire mRNA cap formation in VSV evolves a mechanism distinct to hosts. Thus, mRNA cap modification is an ideal target for vaccine and antiviral drug development. However, there are many questions need to be addressed. It is not known how polymerase coordinates nucleotide polymerization, mRNA cap addition, cap methylation, and polyadenylation. Although the general mechanism of mRNA modifications is defined, the detailed step in each reaction is still poorly understood. The GTPase required for mRNA capping has not been mapped in L protein. Besides the HR motif, it is not known which step of mRNA capping was affected by other mutations (such as T1157A and G1154A) in CR V of L protein. During mRNA cap methylation the exact mechanism by which the dual MTase methylates the 2'-O and G-N-7 is not known. The crystal structure is not known for L protein or any portion of L protein. The concept of using mRNA cap formation as antiviral target has been experimentally demonstrated, however, high-throughput screening methods toward drug discovery have not been developed. Furthermore, there is urgent need to understand the mRNA cap modification in other NNS RNA virus although it is speculated that they may also be achieved in a similar mechanism.

Acknowledgements

Work in Dr. Jianrong Li's laboratory was supported by grants from NIH/NIAID (R01AI090060), USDA Agriculture and Food Research Initiative (2010-65119-20602), and OSU Center for Clinical and Translational Science (CCTS). Yu Zhang is a fellow of Center for RNA Biology at The Ohio State University. We thank Erin DiCaprio for critical review of this manuscript.

Author details

Jianrong Li[1,2,3*] and Yu Zhang[1]

*Address all correspondence to: li.926@osu.edu

1 Department of Food Science and Technology, College of Food, Agricultural and Environmental Sciences, The Ohio State University, USA

http://dx.doi.org/10.5772/54598

2 Division of Environmental Health Sciences, College of Public Health, The Ohio State University, USA

3 Center for RNA Biology, The Ohio State University, Columbus, Ohio, USA

References

[1] Collins PL, Graham BS. Viral and host factors in human respiratory syncytial virus pathogenesis. Journal of virology. 2008;82(5):2040-55.

[2] Falsey AR. Human metapneumovirus infection in adults. The Pediatric infectious disease journal. 2008;27(10 Suppl):S80-3.

[3] Sato M, Wright PF. Current status of vaccines for parainfluenza virus infections. The Pediatric infectious disease journal. 2008;27(10 Suppl):S123-5.

[4] Bloom BR. Vaccines for the Third World. Nature. 1989 Nov 9;342(6246):115-20.

[5] Alamares JG, Li J, Iorio RM. Monoclonal antibody routinely used to identify avirulent strains of Newcastle disease virus binds to an epitope at the carboxy terminus of the hemagglutinin-neuraminidase protein and recognizes individual mesogenic and velogenic strains. Journal of clinical microbiology. 2005;43(8):4229-33.

[6] Abraham G, Rhodes DP, Banerjee AK. The 5' terminal structure of the methylated mRNA synthesized *in vitro* by vesicular stomatitis virus. Cell. 1975;5:51-8.

[7] Abraham G, Rhodes DP, Banerjee AK. Novel initiation of RNA synthesis *in vitro* by vesicular stomatitis virus. Nature. 1975;255(5503):37-40.

[8] Banerjee AK, Rhodes DP. *In vitro* synthesis of RNA that contains polyadenylate by virion-associated RNA polymerase of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America. 1973;70(12):3566-70.

[9] Furuichi Y, Miura KI. A blocked structure at the 5' terminus of mRNA from cytoplasmic polyhedrosis virus. Nature. 1975;253(5490):374-5.

[10] Furuichi Y, Morgan M, Shatkin AJ, Jelinek W, Saldittgeorgieff M, Darnell JE. Methylated, blocked 5 termini in HeLa cell mRNA. Proceedings of the National Academy of Sciences of the United States of America. 1975;72(5):1904-8.

[11] Furuichi Y, Shatkin AJ. Viral and cellular mRNA capping: past and prospects. Adv Virus Res. 2000;55:135-84.

[12] Ogino T, Banerjee AK. Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. Mol Cell. 2007;25:85-97.

[13] Ogino T, Yadav SP, Banerjee AK. Histidine-mediated RNA transfer to GDP for unique mRNA capping by vesicular stomatitis virus RNA polymerase. Proceedings

of the National Academy of Sciences of the United States of America. 2010;107(8): 3463-8.

[14] Li J, Fontaine-Rodriguez EC, Whelan SP. Amino acid residues within conserved domain VI of the vesicular stomatitis virus large polymerase protein essential for mRNA cap methyltransferase activity. Journal of virology. 2005;79(21):13373-84.

[15] Li J, Rahmeh A, Brusic V, Whelan SPJ. Opposing Effects of Inhibiting Cap Addition and Cap Methylation on Polyadenylation during Vesicular Stomatitis Virus mRNA Synthesis. Journal of virology. 2009;83(4):1930-40.

[16] Li J, Rahmeh A, Morelli M, Whelan SP. A conserved motif in region v of the large polymerase proteins of nonsegmented negative-sense RNA viruses that is essential for mRNA capping. Journal of virology. 2008;82(2):775-84.

[17] Li JR, Wang JT, Whelan SPJ. A unique strategy for mRNA cap methylation used by vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(22):8493-8.

[18] Rahmeh AA, Li J, Kranzusch PJ, Whelan SPJ. Ribose 2 '-O Methylation of the Vesicular Stomatitis Virus mRNA Cap Precedes and Facilitates Subsequent Guanine-N-7 Methylation by the Large Polymerase Protein. Journal of virology. 2009;83(21): 11043-50.

[19] Grdzelishvili VZ, Smallwood S, Tower D, Hall RL, Hunt DM, Moyer SA. A single amino acid change in the L-polymerase protein of vesicular stornatitis virus completely abolishes viral mRNA cap methylation. Journal of virology. 2005;79(12): 7327-37.

[20] Whelan SPJ, Barr JN, Wertz GW. Transcription and replication of nonsegmented negative-strand RNA viruses. Biology of Negative Strand RNA Viruses: The Power of Reverse Genetics. 2004;283:61-119.

[21] Abraham G, Banerjee AK. Sequential transcription of the genes of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America. 1976;73(5):1504-8.

[22] Ball LA. Transcriptional mapping of vesicular stomatitis virus in vivo. Journal of virology. 1977;21(1):411-4.

[23] Ball LA, White CN. Order of transcription of genes of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America. 1976;73(2):442-6.

[24] Emerson SU, Wagner RR. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. Journal of virology. 1972;10(2):297-309.

[25] Emerson SU, Yu YH. Both NS and L proteins are required for *in vitro* RNA synthesis by vesicular stomatitis virus. Journal of virology. 1975;15(6):1348-56.

[26] Szilagyi JF, Uryvayev L. Isolation of an infectious ribonucleoprotein from vesicular stomatitis virus containing an active RNA transcriptase. Journal of virology. 1973;11(2):279-86.

[27] Gaudin Y, Barge A, Ebel C, Ruigrok RW. Aggregation of VSV M protein is reversible and mediated by nucleation sites: implications for viral assembly. Virology. 1995;206(1):28-37.

[28] Hammond C, Helenius A. Folding of VSV G protein: sequential interaction with BiP and calnexin. Science. 1994;266(5184):456-8.

[29] Matlin KS, Reggio H, Helenius A, Simons K. Pathway of vesicular stomatitis virus entry leading to infection. Journal of molecular biology. 1982;156(3):609-31.

[30] Follett EA, Pringle CR, Wunner WH, Skehel JJ. Virus replication in enucleate cells: vesicular stomatitis virus and influenza virus. Journal of virology. 1974;13(2):394-9.

[31] Stillman EA, Whitt MA. Transcript initiation and 5 '-end modifications are separable events during vesicular stomatitis virus transcription. Journal of virology. 1999;73(9): 7199-209.

[32] Wang JT, McElvain LE, Whelan SPJ. Vesicular stomatitis virus mRNA capping machinery requires specific cis-acting signals in the RNA. Journal of virology. 2007;81(20):11499-506.

[33] Barr JN, Whelan SP, Wertz GW. cis-Acting signals involved in termination of vesicular stomatitis virus mRNA synthesis include the conserved AUAC and the U7 signal for polyadenylation. Journal of virology. 1997;71(11):8718-25.

[34] Hwang LN, Englund N, Pattnaik AK. Polyadenylation of vesicular stomatitis virus mRNA dictates efficient transcription termination at the intercistronic gene junctions. Journal of virology. 1998;72(3):1805-13.

[35] Poch O, Blumberg BM, Bougueleret L, Tordo N. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. Journal of General Virology. 1990;71:1153-62.

[36] Baltimore D, Huang AS, Stampfer M. Ribonucleic acid synthesis of vesicular stomatitis virus, II. An RNA polymerase in the virion. Proceedings of the National Academy of Sciences of the United States of America. 1970;66(2):572-6.

[37] Rahmeh AA, Schenk AD, Danek EI, Kranzusch PJ, Liang B, Walz T, et al. Molecular architecture of the vesicular stomatitis virus RNA polymerase. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(46):20075-80.

[38] Chattopadhyay A, Raha T, Shaila MS. Effect of single amino acid mutations in the conserved GDNQ motif of L protein of Rinderpest virus on RNA synthesis *in vitro* and in vivo. Virus research. 2004;99(2):139-45.

[39] Smallwood S, Hovel T, Neubert WJ, Moyer SA. Different substitutions at conserved amino acids in domains II and III in the Sendai L RNA polymerase protein inactivate viral RNA synthesis. Virology. 2002;304(1):135-45.

[40] Sleat DE, Banerjee AK. Transcriptional activity and mutational analysis of recombinant vesicular stomatitis virus RNA polymerase. Journal of virology. 1993;67(3): 1334-9.

[41] Chandrika R, Horikami SM, Smallwood S, Moyer SA. Mutations in conserved domain I of the Sendai virus L polymerase protein uncouple transcription and replication. Virology. 1995;213(2):352-63.

[42] Smallwood S, Easson CD, Feller JA, Horikami SM, Moyer SA. Mutations in conserved domain II of the large (L) subunit of the Sendai virus RNA polymerase abolish RNA synthesis. Virology. 1999;262(2):375-83.

[43] Ogino T, Kobayashi M, Iwama M, Mizumoto K. Sendai virus RNA-dependent RNA polymerase L protein catalyzes cap methylation of virus-specific mRNA. Journal of Biological Chemistry. 2005;280(6):4429-35.

[44] Duprex WP, Collins FM, Rima BK. Modulating the function of the measles virus RNA-dependent RNA polymerase by insertion of green fluorescent protein into the open reading frame. Journal of virology. 2002;76(14):7322-8.

[45] Ruedas JB, Perrault J. Insertion of enhanced green fluorescent protein in a hinge region of vesicular stomatitis virus L polymerase protein creates a temperature-sensitive virus that displays no virion-associated polymerase activity *in vitro*. Journal of virology. 2009;83(23):12241-52.

[46] Grdzelishvili VZ, Smallwood S, Tower D, Hall RL, Hunt DM, Moyer SA. Identification of a new region in the vesicular stomatitis virus L polymerase protein which is essential for mRNA cap methylation. Virology. 2006;350(2):394-405.

[47] Rahmeh AA, Morin B, Schenk AD, Liang B, Heinrich BS, Brusic V, et al. Critical phosphoprotein elements that regulate polymerase architecture and function in vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(36):14628-33.

[48] Shuman S. Structure, mechanism, and evolution of the mRNA capping apparatus. Progress in Nucleic Acid Research and Molecular Biology. 2001;66:1-40.

[49] Mizumoto K, Kaziro Y. Messenger RNA capping enzymes from eukaryotic cells. Progress in Nucleic Acid Research and Molecular Biology. 1987;34:1-28.

[50] Venkatesan S, Moss B. Eukaryotic mRNA capping enzyme-guanylate covalent intermediate. Proceedings of the National Academy of Sciences of the United States of America. 1982;79(2):340-4.

[51] Sutton G, Grimes JM, Stuart DI, Roy P. Bluetongue virus VP4 is an RNA-capping assembly line. Nature Structural & Molecular Biology. 2007;14(5):449-51.

http://dx.doi.org/10.5772/54598

[52] Shuman S, Hurwitz J. Mechanism of mRNA capping by vaccinia virus guanylyltransferase: characterization of an enzyme--guanylate intermediate. Proceedings of the National Academy of Sciences of the United States of America. 1981;78(1):187-91.

[53] Cleveland DR, Zarbl H, Millward S. Reovirus guanylyltransferase is L2 gene product lambda 2. Journal of virology. 1986;60(1):307-11.

[54] Furuichi Y, Muthukrishnan S, Tomasz J, Shatkin AJ. Mechanism of formation of reovirus mRNA 5'-terminal blocked and methylated sequence, m7GpppGmpC. Journal of Biological Chemistry. 1976;251(16):5043-53.

[55] Reinisch KM, Nibert M, Harrison SC. Structure of the reovirus core at 3.6 angstrom resolution. Nature. 2000;404(6781):960-7.

[56] Henderson BR, Saeedi BJ, Campagnola G, Geiss BJ. Analysis of RNA binding by the dengue virus NS5 RNA capping enzyme. PloS one. 2011;6(10):e25795.

[57] Luongo CL, Reinisch KM, Harrison SC, Nibert ML. Identification of the guanylyltransferase region and active site in reovirus mRNA capping protein lambda2. The Journal of biological chemistry. 2000;275(4):2804-10.

[58] Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, et al. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature. 2009;458(7240):914-8.

[59] Mir MA, Duran WA, Hjelle BL, Ye C, Panganiban AT. Storage of cellular 5' mRNA caps in P bodies for viral cap-snatching. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(49):19294-9.

[60] Ahola T, Kaariainen L. Reaction in alphavirus mRNA capping: formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:507-11.

[61] Barik S. The structure of the 5' terminal cap of the respiratory syncytial virus mRNA. Journal of General Virology. 1993;74:485-90.

[62] Gupta KC, Roy P. Alternate capping mechanisms for transcription of spring viremia of carp virus: evidence for independent mRNA initiation. Journal of virology. 1980;33(1):292-303.

[63] Liuzzi M, Mason SW, Cartier M, Lawetz C, McCollum RS, Dansereau N, et al. Inhibitors of respiratory syncytial virus replication target cotranscriptional mRNA guanylylation by viral RNA-dependent RNA polymerase. Journal of virology. 2005;79(20): 13105-15.

[64] Ogino T, Banerjee AK. The HR motif in the RNA-dependent RNA polymerase L protein of Chandipura virus is required for unconventional mRNA-capping activity. Journal of General Virology. 2010;91:1311-4.

[65] Cong P, Shuman S. Methyltransferase and subunit association domains of vaccinia virus mRNA capping enzyme. The Journal of biological chemistry. 1992;267:16424-9.

[66] Fabrega C, Hausmann S, Shen V, Shuman S, Lima CD. Structure and mechanism of mRNA cap (guanine-N7) methyltransferase. Molecular Cell. 2004;13(1):77-89.

[67] Hager J, Staker BL, Bugl H, Jakob U. Active site in RrmJ, a heat shock-induced methyltransferase. Journal of Biological Chemistry. 2002;277(44):41978-86.

[68] Hodel AE, Quiocho FA, Gershon PD. VP39 - an mRNA cap-specific 2'-O-methyltransferase. In: X.D. Cheng RMB, editor. S-adenosylmethionine-dependent methyltransferase:structures and functions: World Scientific Publishing; 1999. p. 255-82.

[69] De lPM, Kyrieleis OJP, Cusack S. Structural insights into the mechanism and evolution of the vaccinia virus mRNA cap N7 methyl-transferase. EMBO J. 2007;26:4913-25.

[70] Hodel AE, Gershon PD, Shi XN, Quiocho FA. The 1.85 angstrom structure of vaccinia protein VP39: A bifunctional enzyme that participates in the modification of both mRNA ends. Cell. 1996;85(2):247-56.

[71] Luongo CL, Contreras CM, Farsetta DL, Nibert ML. Binding site for S-adenosyl-L-methionine in a central region of mammalian reovirus lambda2 protein. Evidence for activities in mRNA cap methylation. The Journal of biological chemistry. 1998;273(37):23773-80.

[72] Ray D, Shah A, Tilgner M, Guo Y, Zhao Y, Dong H, et al. West nile virus 5'-cap structure is formed by sequential guanine N-7 and ribose 2'-O methylations by nonstructural protein 5. Journal of virology. 2006;80:8362-70.

[73] Dong H, Ren S, Zhang B, Zhou Y, Puig-Basagoiti F, Li H, et al. West nile virus methyltransferase catalyzes two methylations of the viral RNA cap through a substrate-repositioning mechanism. Journal of virology. 2008;82(9):4295-307.

[74] Egloff M-P, Decroly E, Malet H, Selisko B, Benarroch D, Ferron F, et al. Structural and Functional Analysis of Methylation and 5'-RNA Sequence Requirements of Short Capped RNAs by the Methyltransferase Domain of Dengue Virus NS5. J Mol Biol. 2007;372:723-36.

[75] Martin JL, McMillan FM. SAM (dependent) I AM: the S-adenosylmethionine-dependent methyltransferase fold. Curr Opin Struct Biol. 2002;12:783-93.

[76] Bugl H, Fauman EB, Staker BL, Zheng FH, Kushner SR, Saper MA, et al. RNA methylation under heat shock control. Molecular Cell. 2000;6(2):349-60.

[77] Hager J, Staker BL, Jakob U. Substrate binding analysis of the 23S rRNA methyltransferase RrmJ. Journal of Bacteriology. 2004;186(19):6634-42.

[78] Zhou Y, Ray D, Zhao Y, Dong H, Ren S, Li Z, et al. Structure and function of flavivirus NS5 methyltransferase. Journal of virology. 2007;81:3891-903.

[79] Hu G, Gershon PD, Hodel AE, Quiocho FA. mRNA cap recognition: dominant role of enhanced stacking interactions between methylated bases and protein aromatic

http://dx.doi.org/10.5772/54598

side chains. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(13):7149-54.

[80] Decroly E, Imbert I, Coutard B, Bouvet M, Selisko B, Alvarez K, et al. Coronavirus nonstructural protein 16 is a cap-0 binding enzyme possessing (nucleoside-2'O)-methyltransferase activity. Journal of virology. 2008;82(16):8071-84.

[81] Dong H, Ray D, Ren S, Zhang B, Puig-Basagoiti F, Takagi Y, et al. Distinct RNA elements confer specificity to flavivirus RNA cap methylation events. Journal of virology. 2007;81:4412-21.

[82] Dong H, Ren S, Li H, Shi P-Y. Separate molecules of West Nile virus methyltransferase can independently catalyze the N7 and 2'-O methylations of viral RNA cap. Virology. 2008;377:1-6.

[83] Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK. Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell. 1997;89(6):951-61.

[84] Zhang X, Wei Y, Ma Y, Hu S, Li J. Identification of aromatic amino acid residues in conserved region VI of the large polymerase of vesicular stomatitis virus is essential for both guanine-N-7 and ribose 2'-O methyltransferases. Virology. 2010;408(2): 241-52.

[85] Testa D, Banerjee AK. Two methyltransferase activities in the purified virions of vesicular stomatitis virus. Journal of virology. 1977;24(3):786-93.

[86] Li J, Chorba JS, Whelan SP. Vesicular stomatitis viruses resistant to the methylase inhibitor sinefungin upregulate RNA synthesis and reveal mutations that affect mRNA cap methylation. Journal of virology. 2007;81(8):4104-15.

[87] Horikami SM, Moyer SA. Host range mutants of vesicular stomatitis virus defective in *in vitro* RNA methylation. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences. 1982;79(24):7694-8.

[88] Issur M, Geiss BJ, Bougie I, Picard-Jean F, Despins S, Mayette J, et al. The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. RNA. 2009;15:2340-50.

[89] Koonin EV, Moss B. Viruses know more than one way to don a cap. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(8): 3283-4.

[90] Colonno RJ, Stone HO. Newcastle disease virus mRNA lacks 2'-O-methylated nucleotides. Nature. 1976 1976;261(5561):611-4. PubMed PMID: WOS:A1976BU52600052.

[91] Murphy AM, Grdzelishvili VZ. Identification of sendai virus L protein amino acid residues affecting viral mRNA cap methylation. Journal of virology. 2009;83(4): 1669-81.

[92] Murphy AM, Moerdyk-Schauwecker M, Mushegian A, Grdzelishvili VZ. Sequence-function analysis of the Sendai virus L protein domain VI. Virology. 2010;405(2): 370-82.

[93] Hunt DM. Vesicular stomatitis virus mutant with altered polyadenylic acid polymerase activity *in vitro*. Journal of virology. 1983;46(3):788-99.

[94] Hunt DM. Effect of analogues of S-adenosylmethionine on *in vitro* polyadenylation by vesicular stomatitis virus. The Journal of general virology. 1989;70 (Pt 3):535-42.

[95] Hunt DM, Mehta R, Hutchinson KL. The L protein of vesicular stomatitis virus modulates the response of the polyadenylic acid polymerase to S-adenosylhomocysteine. The Journal of general virology. 1988;69 (Pt 10):2555-61.

[96] Rose JK, Lodish HF, Brock ML. Giant heterogeneous polyadenylic acid on vesicular stomatitis virus mRNA synthesized *in vitro* in the presence of S-adenosylhomocysteine. Journal of virology. 1977;21(2):683-93.

[97] Galloway SE, Wertz GW. S-adenosyl homocysteine-induced hyperpolyadenylation of vesicular stomatitis virus mRNA requires the methyltransferase activity of L protein. Journal of virology. 2008;82(24):12280-90.

[98] Hunt DM, Hutchinson KL. Amino acid changes in the L polymerase protein of vesicular stomatitis virus which confer aberrant polyadenylation and temperature-sensitive phenotypes. Virology. 1993;193(2):786-93.

[99] Hunt DM, Smith EF, Buckley DW. Aberrant polyadenylation by a vesicular stomatitis virus mutant is due to an altered L protein. Journal of virology. 1984;52(2):515-21.

[100] Muthukrishnan S, Both GW, Furuichi Y, Shatkin AJ. 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature. 1975;255(5503):33-7.

[101] Muthukrishnan S, Moss B, Cooper JA, Maxwell ES. Influence of 5'-terminal cap structure on the initiation of translation of vaccinia virus mRNA. The Journal of biological chemistry. 1978;253(5):1710-5.

[102] Lodish HF, Porter M. Translational control of protein synthesis after infection by vesicular stomatitis virus. Journal of virology. 1980;36(3):719-33.

[103] Lodish HF, Porter M. Vesicular stomatitis virus mRNA and inhibition of translation of cellular mRNA--is there a P function in vesicular stomatitis virus? Journal of virology. 1981;38(2):504-17.

[104] Connor JH, Lyles DS. Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1. Journal of virology. 2002;76(20):10177-87.

[105] Qanungo KR, Shaji D, Mathur M, Banerjee AK. Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(16):5952-7.

http://dx.doi.org/10.5772/54598

[106] Whelan SPJ, Wertz GW. Transcription and replication initiate at separate sites on the vesicular stomatitis virus genome. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(14):9178-83.

[107] Ma Y WY, Divers E, Whelan SPJ, Li J. , editor The impact of mRNA cap methylation status on the pathogenesis of vesicular stomatitis virus in vivo. American Soceity for Virology; 2011; Minneapolis, Minnesota, USA.

[108] Sabin AB, Olitsky PK. Influence of Host Factors on Neuroinvasiveness of Vesicular Stomatitis Virus : Iii. Effect of Age and Pathway of Infection on the Character and Localization of Lesions in the Central Nervous System. The Journal of experimental medicine. 1938;67(2):201-28.

[109] Sabin AB, Olitsky PK. Influence of Host Factors on Neuroinvasiveness of Vesicular Stomatitis Virus : Ii. Effect of Age on the Invasion of the Peripheral and Central Nervous Systems by Virus Injected into the Leg Muscles or the Eye. The Journal of experimental medicine. 1937;66(1):35-57.

[110] Sabin AB, Olitsky PK. Influence of Host Factors on Neuroinvasiveness of Vesicular Stomatitis Virus : I. Effect of Age on the Invasion of the Brain by Virus Instilled in the Nose. The Journal of experimental medicine. 1937;66(1):15-34.

[111] Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, et al. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature. 2010;468(7322):452-6.

[112] Zust R, Cervantes-Barragan L, Habjan M, Maier R, Neuman BW, Ziebuhr J, et al. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. Nature immunology. 2011;12(2):137-43.

[113] Wilkins C, Dishongh R, Moore SC, Whitt MA, Chow M, Machaca K. RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. Nature. 2005;436(7053):1044-7.

[114] De Clercq E. Antivirals and antiviral strategies. Nature Reviews Microbiology. 2004;2(9):704-20.

[115] de Clercq E, Montgomery JA. Broad-spectrum antiviral activity of the carbocyclic analog of 3-deazaadenosine. Antiviral research. 1983;3(1):17-24.

[116] Schluckebier G, Zhong P, Stewart KD, Kavanaugh TJ, Abad-Zapatero C. The 2.2 A structure of the rRNA methyltransferase ErmC' and its complexes with cofactor and cofactor analogs: implications for the reaction mechanism. Journal of molecular biology. 1999;289(2):277-91.

IntechOpen