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MINIREVIEW

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Eukaryotic mRNAs possess a blocked 5′ end known as the cap structure. Capping occurs on nascent RNA transcripts and is catalyzed by nuclear enzymes. Most animal viruses that replicate in the cytoplasm of infected cells also possess capping activities and produce capped mRNAs. This modification is involved in pre-mRNA processing and transport from the nucleus, recruitment of mature mRNAs to the ribosomes, and in the protection of mRNAs from degradation (reviewed in: Reddy *et al.,* 1992).

In the cap structure, a guanosine residue blocks the 5′-terminal base of the mRNA via a unique 5′-5′ triphosphate linkage GpppN, where the penultimate "N" nucleoside is derived from the RNA transcript. The blocking guanosine residue of almost all cellular and viral mRNAs contains a methyl group at the N-7 position (m^7GpppN). In small nuclear RNAs, $m^{2,7}$GpppN or $m^{2,2,7}$GpppN cap structures are often found instead of m^7GpppN (Vartapetian and Bogdanov, 1987; Furuichi and Shatkin, 1989; Liou and Blumenthal, 1990; Reddy *et al.,* 1992). Furthermore, in both mRNA and small nuclear RNA, the 5′-terminal penultimate base and its adjoining residue are sometimes methylated in the ribose moiety or in the N-6 position of adenosine when it is found at the penultimate position.

The cap structure is synthesized by three enzymatic reactions in which the 5′-triphosphate terminus of a primary transcript is cleaved to a diphosphate-terminated RNA by an RNA triphosphatase and capped with GMP by an RNA guanylyltransferase. Finally, an RNA (guanine-7-) methyltransferase catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the N-7 position of the blocking guanosine. This pathway seems to apply to all cellular capping enzymes, although few examples have been characterized in details; however, some exceptions to this general mechanism have been described for RNA viruses that will be discussed later in this review. Since the discovery of the cap structure in the 1970s, many studies have been performed to elucidate its structure, synthesis, as well as functional role; various reviews were devoted to this topic (Shatkin, 1976; Filipowicz, 1978; Banerjee, 1980; Reddy *et al.,* 1992). In contrast, this short review focuses on the proteins involved in the capping reaction, many of which have been isolated and characterized only in recent years.

RNA TRIPHOSPHATASES

Since the enzymatic activities required for the synthesis of the cap structure are found in many purified virions, viruses have been extensively used to define the processes involved in cap formation. The cytoplasmic DNA vaccinia virus capping enzyme, a heterodimeric protein composed of 95- and 33-kDa subunits, is encoded by the viral D1 and D12 genes and has been extensively analyzed (Martin *et al.,* 1975). This protein complex is able to perform all the reactions required for the synthesis of the cap structure since it has RNA triphosphatase, guanylytransferase, and methyltransferase activities (Venkatesan *et al.,* 1980). The triphosphatase and guanylyltransferase reactions are catalyzed by the amino-terminal 60 kDa of the D1 subunit. Mutations in this subunit inactivating one activity without affecting the other have been described and this suggests the existence of two distinct functional domains responsible for triphosphatase and guanylyltransferase activities (Cong and Shuman, 1995). Kinetic studies have established that the conversion of triphosphate to diphosphate ends by the multifunctional vaccinia capping enzyme is 100-fold faster than GMP transfer to the RNA acceptor molecules (Venkatesan *et al.,* 1980). This property of the vaccinia enzyme probably ensures that the guanylyltransferase has little or no exposure at all to triphosphate-terminated RNAs. This is of importance since it has recently been observed that two vaccinia virus RNA triphosphatase-defective mutants can transfer GMP to 5′-triphosphate

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RNA ends to produce an unusual tetraphosphate cap structure GppppA . . . (Yu and Shuman, 1996). Synthesis of small amounts of free dinucleoside tetraphosphate has been previously reported during *in vitro* transcription with cytoplasmic polyhedrosis virus, a dsRNA virus of the *Reoviridae* family, and vaccinia virus (Smith and Furuichi, 1982). Studies have also demonstrated that the guanylyltransferases from avian reovirus and yeast *Saccharomyces cerevisiae* can transfer the GMP moiety to a GTP acceptor yielding GppppG (Martinez-Costas *et al.,* 1995; Wang and Shatkin, 1984). The biological significance, if any, of tetraphosphate capping is not currently known and there is still no evidence that formation of tetraphosphate caps occurs during transcription of viral mRNAs *in vivo.* However, dinucleoside tetraphosphates (GppppG and AppppA) were found *in vivo* in several mammalian cells and in embryos of other eukaryotic organisms, suggesting that tetraphosphate capping by cellular enzymes occurs to a certain extent (Finamore and Warner, 1963; Oikawa and Smith, 1966; Rapaport and Zamecnik, 1976). All these observations stress the importance of RNA triphosphatase for the synthesis of usual triphosphorylated cap structure.

The RNA triphosphatase component of the vaccinia virus capping enzyme hydrolyzes the γ-phosphate of triphosphate-terminated RNA and can also cleave the γ-phosphate out of free nucleoside triphosphates. Competitive inhibition and simultaneous mutational inactivation of RNA triphosphatase and nucleoside triphosphatase functions suggest that both reactions occur at a single active site (Yu and Shuman, 1996; Myette and Niles, 1996b). However, the observation that the K_m for ATP is 3 orders of magnitude higher than for triphosphate-terminated poly(A) suggests that RNA is the preferred, and most likely physiological, substrate (Myette and Niles, 1996a).

An RNA triphosphatase activity has been assigned to the NS3 protein of the West Nile virus (flavivirus, single-stranded RNA) (Wengler and Wengler, 1993). As for the vaccinia virus enzyme, the NS3 protein is also a multifunctional protein; it possesses a protease activity in its amino-terminal part, a helicase activity in the central region, and the RNA triphosphatase tentatively assigned to the carboxy-terminal domain (Wengler *et al.,* 1991; Tai *et al.,* 1996; Wengler and Wengler, 1993). It has been noted that the LRPR amino acid sequence found in this West Nile Virus protein is similar to the vaccinia virus D1 subunit sequence LKPR. Since no actual structure–function studies have been performed, the importance of this motif remains purely speculative, although a similar motif (LRIR) is also present in the reovirus λ1 protein to which nucleoside and RNA triphosphate phosphohydrolase activities were recently assigned (Bartlett and Joklik, 1988; Noble and Nibert, 1997; Bisaillon *et al.,* 1997 and unpublished data). Furthermore, another somewhat degenerate motif seems to be shared by the vaccinia virus capping enzyme D1 subunit (RPNTSLE), West Nile virus NS3 (RTNTILE), and reovirus λ1 protein (RDETGLM). These motifs are also found on various putative RNA triphosphatases of other flaviviruses and DNA viruses. Interestingly, a substitution of the glutamate residue in this latter motif of the vaccinia virus capping enzyme inactivates the triphosphatase but does not affect the guanylyltransferase activity (Yu and Shuman, 1996). This supports the idea that these consensus motifs have a functional significance, although further studies will be needed to firmly establish their exact nature and importance.

Early attempts to demonstrate the 5′-RNA triphosphatase activity in cellular extracts were unsuccessful mainly due to the abundant presence of contaminant, nonspecific, nucleotide phosphohydrolase activities (Yagi *et al.,* 1983). More recently, numerous improvements in protein purification techniques and the use of recombinant technology have allowed the identification of RNA triphosphatases from a variety of sources. Purified cellular RNA triphosphatases were isolated from rat liver cells, the brine shrimp *Artemia salina,* and the yeast *S. cerevisiae* (Yagi *et al.,* 1983, 1984; Itoh *et al.,* 1984). These RNA triphosphatases are all specific to the removal of 5′-γ-phosphate from RNA chains without attacking the β-phosphoryl group. Further studies revealed that the native yeast capping enzyme has both RNA triphosphatase and guanylyltransferase activities (Itoh *et al.,* 1987). However, these activities reside on separate polypeptides, encoded by different genes (Shibagaki *et al.,* 1992). In contrast, all attempts to separate the two activities of the shrimp or rat enzymes were unsuccessful and it appears that both activities are exerted by a single polypeptide chain, although proteolytic digestion can separate the functional domains of the brine shrimp protein (Yagi *et al.,* 1984).

RNA GUANYLYLTRANSFERASES

Viral-encoded guanylyltransferases have been extensively studied. The formation of a covalent enzyme-guanylate intermediate has been identified in guanylyltransferases isolated from a variety of viruses. In the majority of cases, the enzyme catalyzes the transfer of a GMP residue from GTP to the 5′-diphosphoryl-terminated mRNAs following removal of the γ-phosphate by RNA triphosphatase. The GMP is covalently linked to the RNA guanylyltransferase through a phosphoamide bond to the ϵ-amino group of a lysine residue (Roth and Hurwitz, 1984). A first exception to this general mechanism has been noted in the formation of the vesicular stomatitis virus cap structure. Although the mechanistic details are not currently understood, the GpppA . . . cap is formed from the α- and β-phosphates of a GTP molecule and

from a monophosphorylated RNA acceptor, in contrast to the conventional pathway in which α- and β-phosphates are derived from the RNA molecule. Furthermore, there is no apparent binding of guanine nucleotides (GTP, GDP, GMP) to any viral polypeptides, raising the possibility of a completely different mechanism of cap formation (reviewed in: Shuman, 1997). Another apparent exception is the birnavirus bursal disease virus p90 protein, a putative guanylyltransferase identified through its ability to form an enzyme-guanylate intermediate, although no actual transfer of the GMP to the RNA acceptor molecule has been demonstrated (Spies and Müller, 1990); in this case, a phosphodiester linkage seems to be present between the GMP and a serine or threonine residue of the protein.

Compared to RNA triphosphatases, determination of the primary structure of RNA guanylyltransferases from many viruses, as well as *S. cerevisiae,* has allowed a better knowledge of the active site and catalytic mechanism. A lysine-containing motif, KxDG, is conserved among guanylyltransferases encoded by DNA viruses (vaccinia virus, Shope fibroma virus, African swine fever virus) and the yeasts *S. cerevisiae* and *S. pombe* (Cong and Shuman, 1993; Shuman *et al.,* 1994). This motif is also conserved at the active site of polynucleotide ligases which, like capping enzymes, catalyze an enzymatic reaction via the formation of a covalent Lys-nucleoside monophosphate intermediate (Lindahl and Barnes, 1992). On the basis of mutational analysis and peptide mapping, Lys-260 within the KTDG motif of the vaccinia guanylyltransferase was defined as the active site residue involved in radioactive GMP covalent binding; similar approaches also identified Lys-70 within the KTDG motif of the yeast capping enzyme (Cong and Shuman, 1993; Schwer and Shuman, 1994). Furthermore, since the yeast guanylyltransferase is essential for cell growth, it is possible to test mutational effects on enzyme function *in vivo;* conservative (Arg) or nonconservative (Ala) substitution of Lys-70 abrogates capping enzyme function *in vivo* as judged by viability loss (Shuman *et al.,* 1994). Substitution of the invariant Gly residue with Ala is also lethal, as expected since this mutation abolished protein-GMP formation *in vitro,* as also observed in the vaccinia virus enzyme. In addition to the active guanylyltransferase site (KxDG), other conserved sequence elements (II, III, IV, and V) were also identified in enzymes from DNA viruses (vaccinia virus, Shope fibroma virus, African swine fever virus), *S. cerevisiae, S. pombe,* and among members of the ATP-dependent ligase family (Shuman *et al.,* 1994). These elements are all arranged in the same order and with nearly identical spacing, although their sequence can be slightly divergent. Individual residues in motifs III, IV, and V of the yeast capping enzyme were shown to be essential for capping enzyme function *in vivo.* Substitution of the highly conserved Glu residue in motif III of the *S. cerevisiae* capping enzyme is lethal *in vivo* and specific residues in motif IV (Asp-225 and Gly-226) and V (Lys-249 and Asp-257) are also essential (Shuman *et al.,* 1994). Similarly, mutational analysis of the vaccinia virus guanylyltransferase has established that specific residues in motifs III, IV, and V are important for GTP binding and GMP transfer from the enzyme to the RNA acceptor (Cong and Shuman, 1995). Thus, the conserved motifs in addition to the GMP binding lysine *per se* are important for enzyme function and this suggests a common structural basis for covalent nucleotidyl transfer by these enzymes.

The mRNAs of alphaviruses (positive-strand RNA viruses; e.g., Sindbis and Semliki Forest virus) harbor a cap structure synthesized by the nsP1 protein that has both guanylyltransferase and (guanosine-7-) methyltransferase activities. However, it was recently demonstrated that the guanine nucleotide bound to nsP1 is exclusively m^7GMP, instead of the usual GMP (Ahola and Kääriäinen, 1995). This property is unique to the alphaviruses nsP1 protein as all other known guanylyltransferases (vaccinia virus, reovirus, HeLa cells, rat liver cells, and *S. cerevisiae*) are only able to form a linkage with GMP (and dGMP) and not m^7GMP. It was therefore proposed that methylation of GTP by nsP1 precedes covalent adduct formation and that m^7GTP is the true substrate donor for the guanylyltransferase reaction (Ahola and Kääriäinen, 1995). This suggests that the unique nsP1-m^7GMP complex could be a target in designing drugs that specifically inhibit the capping of alphavirus mRNAs. It is worth mentioning that the characteristically conserved motifs found in guanylyltransferases of DNA viruses are not found in the sequence of alphavirus nsP1 protein from various isolates. A similar situation is also observed with the reovirus λ2 protein, rotavirus VP3, and bluetongue virus VP4, all viral guanylyltransferases from viruses of the *Reoviridae* family (Seliger *et al.,* 1987; Mitchell and Both, 1990; Yu *et al.,* 1987). These enzymes form a covalent enzyme-guanylate intermediate but lack the conserved motifs found in yeast and DNA virus guanylyltransferases. These enzymes apparently evolved differently from other viral and cellular guanylyltransferases despite their identical enzymatic functions. The sequence at the active site of reovirus λ2 protein, KPTNG, diverges from the consensus signature of DNA viruses (KxDG) but is related to the murine rotavirus SA-11 VP3 protein sequence (KPTGN) and bluetongue virus VP4 (KLTGD); the Lys(226) residue of the reovirus λ2 protein KPTGN sequence was actually identified as the GMP attachment site (Fausnaugh and Shatkin, 1990). Furthermore, another motif seems to be conserved between the reovirus λ2 protein (YVRKN), the VP3 protein of the murine rotavirus SA-11 (YYRYN), and the bluetongue virus VP4 (YKRKM). Mutagenesis of these residues could eventually reveal important amino acids involved in guanylate binding or GMP transfer from the

enzyme to the acceptor mRNA molecules in this class of proteins.

METHYLTRANSFERASES

Methyltransferases are the third key enzymes in the formation of the cap structure. Early biochemical studies have focused primarily on the native heterodimeric vaccinia virus capping enzyme which, as stated previously, also catalyzes the (guanosine-7-) methylation. It has been shown that the carboxy-terminal region of the D1 subunit has a low intrinsic methyltransferase activity, which can be stimulated by heterodimerization with the D12 subunit (Mao and Shuman, 1994). Furthermore, binding studies have reveal that the C-terminal region of the D1 protein can bind RNA, *S*-adenosylmethionine (methyl donor), and GTP (methyl acceptor). Homologous amino acids regions are found in the putative methyltransferases of two other DNA viruses, Shope fibroma virus, and African swine fever virus. Mutational analysis revealed that a conserved His-Tyr motif of the vaccinia virus capping enzyme is likely a component of the active site since substitution of these amino acids abolished the methyltransferase activity (Mao and Shuman, 1994).

The study of additional viruses has provided further insights into the role of the methyltransferases *in vivo.* Many viral methyltransferases, as in vaccinia virus, have an additional polymerase or guanylyltransferase domain. This feature distinguishes them from most cellular proteins, which typically appear to have only methyltransferase activity (Reddy *et al.,* 1992).

The nsP1 proteins of Semliki Forest virus and Sindbis virus, two positive-strand RNA viruses, also catalyze a methyltransferase reaction along with their RNA guanylyltransferase activity (Mi *et al.,* 1989; Laakkonen *et al.,* 1994). Substitution of various conserved amino acid residues of the nsP1 proteins of these two related viruses abolish or greatly reduce the methyltransferase activity (Wang *et al.,* 1996; Ahola *et al.,* 1997). Not surprisingly, all the methyltransferase-negative mutants also loose the guanylyltransferase activity, confirming that methylation of GTP is an essential prerequisite for the synthesis of the covalent guanylate complex in this particular virus family (Ahola and Kääriäinen, 1995). These mutations also lead to a loss of viral infectivity, again supporting the essential role of mRNA capping. Further evidence also came from studies of a mutant Sindbis virus in which changes in the nsP1 protein result in an ability to multiply under reduced methionine concentration. This novel property results from an enhanced affinity of the mutant methyltransferase for *S*-adenosylmethionine, the methyl-donor derivative of methionine (Scheidel *et al.,* 1989).

The amino acid sequence of different viral and cellular methyltransferases are widely variable but a conserved sequence element, the G-loop UU[D/E]UooGxo, can be defined; where U designates a bulky hydrophobic amino acid residue, o represents a small residue (G, A, or S), and x is any amino acid (Ingrosso *et al.,* 1989). The importance of the G-loop in binding of the methyl donor (*S*-adenosylmethionine) has been suggested by mutational analysis of a DNA methyltransferase, an unrelated enzyme that nevertheless uses *S*-adenosylmethionine as a methyl donor for DNA methylation (Willcock *et al.,* 1994).

The G-loop is found in the N-terminal-portion of the NS5 protein of different flaviviruses and in the C-terminal portion of the λ2 protein of reovirus, although no actual enzymatic activity has been demonstrated for these different proteins (Koonin, 1993). The NS5 protein of flaviviruses is a two-domain protein, with its C-terminal domain comprising the RNA-dependent polymerase. The reovirus λ2 protein has guanylyltransferase activity, mapped to the N-terminal region of the protein. Isolated λ2 failed to methylate the cap of reovirus mRNA and it has been suggested that an interaction with other proteins in the virus core may be required for the protein to exert its activity (Mao and Joklik, 1991). Interestingly, the reovirus λ2 protein also harbors 3 additional amino acids motifs (L/IXY, TI/TXXXD/E, EXGXXL/I) shared by the methyltransferases of DNA viruses and *S. cerevisiae* but apparently absent from the flavivirus proteins (Mao *et al.,* 1996). The significance of these motifs still needs to be established.

Cellular RNA (guanine-7-) methyltransferases have been isolated from rat liver, HeLa cells and recently from *S. cerevisiae* (Mizumoto and Lipmann, 1979; Ensinger and Moss, 1976; Mao *et al.,* 1995). The yeast methyltransferase *ABD1* gene was identified on the basis of its sequence similarity with the vaccinia virus methyltransferase and represents the first cellular gene known to encode a cap methyltransferase (Mao *et al.,* 1995). Deletion of the *ABD1* gene is lethal and all mutations that affect the methyltransferase activity *in vitro* are either lethal or severely affect cell growth, indicating that cap methylation is an essential function *in vivo* (Mao *et al.,* 1996).

The nucleotides adjacent to the cap structure can also be methylated to different extents and the resulting structures are referred to as "cap 0," "cap 1," or "cap 2," corresponding to the number of methylated sugar residues (Furuichi and Shatkin, 1989). The role of the ribose methylations present in the second and third nucleotide from the 5′-end of many mRNAs remains unknown, although some data indicated that these methylations may have a stimulatory effect on ribosome binding of ribopolymers with weak affinities (Muthukrishnan *et al.,* 1976). In higher eukaryotes, the mRNAs contain mainly cap 2 structures, whereas the mRNAs in lower eukaryotes contain mainly cap 0. An unusual cap 4 structure is also found in *Trypanosoma brucei* where the first four residues are 2′-*O*-methylated (Freistadt *et al.,* 1988). Moreover, when the 5′-penultimate base of viral or cellular mRNAs is adenosine, it exists predominantly in a dimethylated form, N6-

methyl-2′-*O*-methyladenosine. A methyltransferase activity which can methylate specifically the N-6 position of the 2′-*O*-methyladenosine is present in HeLa cells but this enzyme does not methylate internal adenosine residues, suggesting that a separate methyltransferase catalyzes the formation of internal m6A found in many eukaryotic mRNAs (Keith *et al.,* 1978).

RNA 2′-*O*-methyltransferases have been isolated from cellular and viral sources. The vaccinia virus RNA 2′-*O*-methyltransferase catalyzes the transfer of a methyl group to the 2′-OH of the penultimate nucleoside of capped mRNAs (Barbosa and Moss, 1978). Recent data indicate that the activity is mediated by the vaccinia virus VP39, the stimulatory subunit of the poly(A) polymerase (Schnierle *et al.,* 1992; Gershon *et al.,* 1991). The VP39 is thus a bifunctional protein involved in the maturation of both ends of vaccinia virus transcripts; the RNA 2′-*O*-methyltransferase is specific for the capped 5′ end of mRNAs, whereas the poly(A) polymerase stimulatory activity is manifested at the 3′ end. Mutational analysis has identified several regions of VP39 important for interaction with RNA, cap structure, and *S*-adenosylmethionine (Schnierle *et al.,* 1994; Shi *et al.,* 1996). The region between residues 138 and 150 was shown to be important for *S*-adenosylmethionine binding and possibly also for methyltransferase activity (Shi *et al.,* 1996). Furthermore, comparison of the crystal structure of VP39 with prokaryotic and mammalian methyltransferases reveals a very high degree of structural conservation between these enzymes (Hodel *et al.,* 1996).

CONCLUSION

The unique 5′-terminal cap structure of eukaryotic mRNAs clearly differentiates them from polycistronic prokaryotic mRNAs, which harbor unblocked triphosphorylated ends. Although the cap structure was discovered in the 1970s, and its importance in mRNA processing and translation has been amply documented *in vitro,* we are only beginning to understand its crucial role *in vivo.* Genetic analysis of the yeast enzymes involved in cap synthesis appears as a powerful *in vivo* tool to elucidate the critical role of the cap structure. However, it is likely that the study of viruses will continue to significantly contribute to a better understanding of the capping reaction. The recent identification, purification, and analysis of capping enzymes from a wide variety of viral and cellular sources, are important steps toward this goal. Finally, viral capping enzymes offer promising targets for antiviral drug design and therapy since many RNA viruses use unconventional substrates or capping strategies.

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