INTRODUCTION

Most eukaryotic and viral mRNAs contain a 5′-cap structure that is important for RNA stability and translation (Furuichi & Shatkin, 2000). In general, the RNA capping process consists of four steps: (1) hydrolysis of the 5′ triphosphate end of the nascent RNA transcript to a 5′ diphosphate by a 5′ RNA triphosphatase (RTPase), (2) transfer of the GMP moiety of GTP to the 5′ diphosphate of RNA by an RNA guanylyltransferase (GTase), (3) methylation of the N7 position of guanine by an RNA guanine methyltransferase (MTase), yielding a cap 0 structure, and (4) further methylation of the first and second nucleotides of RNA at the ribose 2′-OH position by a nucleoside 2′-O-MTase to form a cap 1 (m7GpppN) and cap 2 (m7GpppNmNm) structure, respectively (Furuichi & Shatkin, 2000). S-adenosyl-L-methionine (SAM) is the methyl group donor for both the N7 and 2′-O-methylation. During virus replication, different viruses employ their own unique mechanism for RNA capping (Benarroch et al., 2009; Koonin & Moss, 2010).

Cytoplasmic polyhedrosis virus belongs to the genus Cypovirus of the family Reoviridae (Mertens et al., 2005) and contains 10 segments of dsRNA (Payne & Mertens, 1983). Each dsRNA segment is composed of a plus strand and its complementary minus strand in an end-to-end base pair configuration except for a protruding 5′-capped end (Furuichi & Miura, 1975). Cypovirus is unique among the dsRNA viruses in having a single-layer capsid (or core) which functions to transcribe mRNA from the minus-strand RNA genome by RNA-dependent RNA polymerase (RdRp) and then to cap nascent mRNA by other viral enzymes before its release into the cytoplasm for the initiation of viral protein translation. The capsid also serves as a shelter protecting this transcriptional process from antiviral defence mechanisms present inside the cytoplasm of host cells (Xia et al., 2003; Zhou et al., 2003).

In the case of cytoplasmic polyhedrosis virus (CPV) infecting the mulberry silkworm Bombyx mori (BmCPV), which belongs to the ‘turreted reoviruses’, the capsid is formed by three major proteins: VP1 (capsid shell protein), VP3 (turret protein) and VP5 (spike-like protein) encoded by its genome segments S1, S4 and S7, respectively. Analysis of the three-dimensional structure of BmCPV by cryo-electron microscopy (cryo-EM) revealed that mRNA capping occurred in the enzymic domains of the pentameric turret whose five unique channels guide nascent mRNA sequentially to GTase, N7-MTase and 2′-O-MTase domains in order to fulfill the highly coordinated mRNA capping activity (Cheng et al., 2011). The most recent
structural comparison via cryo-EM of transcribing and non-transcribing BmCPV showed that transfer of a GMP moiety occurred to the 5'-end of the diphosphate-ended RNA after its binding to Lys234 of the GTase pocket via a phosphoamide linkage (Yang et al., 2012) and then the RNA capping reaction occurred in the active sites of different turret protein monomers (Zhu et al., 2014).

In the case of viruses lacking a pentameric turret, core protein VP3 (in the case of rotavirus) or VP4 [in the case of bluetongue virus (BTV)] provides both GTase and MTase activity for capping the 5'-end of viral RNA (Trask et al., 2012). In many viruses, GTase activity has only been reported to be associated with RTase activity, such as mammalian reoviral µ2 (Kim et al., 2004; Noble & Nibert, 1997b) and λ1 proteins (Bisaillon et al., 1997; Noble & Nibert, 1997a), avian reoviral µA protein (Su et al., 2007), rotaviral NSP2 protein (Vasquez-Del Carpio et al., 2006), dengue viral NS3 protein (Barletma & Padmanabhan, 2002), aquareoviral VP5 protein (Attouli et al., 2002) and alfavirus NSP2 protein (Vesiljeva et al., 2000), which not only has RTase activity but also GTase activity.

For the methylation of the 5’-cap structure, the organization of the MTase domains is different in different viruses. In the case of chlorella virus, the RTase, GTase and N7-MTase are encoded by separate polypeptides (Håkansson et al., 1997); in vaccinia virus (VV), protein D1 possesses N7-MTase activity (De la Peña et al., 2007) and VP39 possesses 2'-O-MTase activity (Hodel et al., 1996); in West Nile virus (WNV), NS5 protein has only one MTase domain catalysing both N7-MTase and 2’-O-MTase reactions through a substrate-repositioning mechanism (Dong et al., 2008; Ray et al., 2006; Zhou et al., 2007), whereas in the case of BTV, protein VP4 is not only responsible for the GTase and RTase activities (Martinez-Costas et al., 1998), but also has MTase activity (Ramadevi et al., 1998).

Antheraea mylitta cytoplasmic polyhedrosis virus (AmCPV) is one of the most widespread pathogens of the Indian non-mulberry silkworm causing significant losses to the silk industry (Jolly et al., 1974). The AmCPV genome consists of 11 segments of dsRNA (S1–S11) (Qanungo et al., 2000). Most of the genome segments except S4 have been cloned, sequenced and characterized functionally. S1 and S3 code for viral capsid proteins (Chakrabarti et al., 2010), S2 codes for RdRp (Ghorai et al., 2010), S6 codes for a protein having ATP-binding and ATPase activity (Chavali et al., 2008), S7 and S8 code for viral structural proteins (Chavali & Ghosh, 2007; Jangam et al., 2006), S9 codes for a non-structural protein having RNA-binding properties (Qanungo et al., 2002), S10 codes for viral polyhedrin (Sinha-Datta et al., 2005), and S11 does not contain any ORF (Jangam et al., 2006). We have reported recently that AmCPV S5 encodes a 65 kDa viral GTase without any RTase activity and catalyses the formation of a RNA cap structure using GTP as a substrate through initial formation of an enzyme–GMP complex via phosphoamide linkage and then transferring the GMP moiety to the diphosphate-ended viral RNA (Biswas et al., 2014). As sequences of AmCPV do not show any homology with BmCPV and the mechanism of capping has not been fully elucidated, especially the methylation of the cap structure, molecular and biochemical characterization of the capping enzymes is necessary.

Here, we report the molecular cloning, sequencing and expression of AmCPV S4 that encodes a 127 kDa protein (p127). Using a biochemical assay, we showed that p127 contained RTase as well as MTase activity which catalysed the removal of the phosphate group from the 5’ end of the viral RNA, and then methylated guanine N7 and 2’-O ribose in GpppG-RNA in a sequential manner to complete the capping process.

**RESULTS**

**Genetic analysis of AmCPV S4**

AmCPV S4 dsRNA was isolated, reverse transcribed to its cDNAs, cloned into pCR-XL-TOPO vector and sequenced. The AmCPV S4 cDNA consisted of 3410 nt with a single ORF of 1110 aa which could encode a protein of ~127 kDa (p127). The S4 ORF started with an ATG codon at nt 45 and ended with the TAA stop codon at nt 3375 (GenBank accession number KJ938018). The theoretical isoelectric point of p127 was calculated as 5.86. The secondary structure prediction using GOR4 showed that 44.68% of residues were likely to form random coils, 34.50% would form α-helices and 20.81% would form extended sheets. BLAST analysis did not show any significant homology of p127 with any other nucleotide or protein sequences in the public databases. Therefore, to assess the possible role of p127 in the viral life cycle, deduced amino acid sequences of p127 were aligned with known conserved motifs of several viral proteins. This type of analysis showed that p127 could be aligned with the RTase motif (LKPR/LRPR/LRIR) of VV, WNV and mammalian orthoreovirus (MRV) (Fig. 1a), and with the SAM-binding domain (GxGxG) of WNV, Japanese encephalitis virus (JEV) and MRV (Fig. 1b). In addition, amino acid sequence alignment also showed that p127 contained Gly387, Gly902 and Asp908 (GGD motif) which overlapped closely in Gly-(X3-4)-Asp-(X11-15)-Asp (GDD motif) of BmCPV, Encephalitozoon cuniculi (Ecm1) and VV N7-MTase (Fig. 1c) as well as the Lys538-Asp622-Lys659-Glu689 (KDKE) tetrad of BmCPV 2’-O-MTase (Fig. 1d). These results indicated that S4 may code for RTase, N7-MTase and 2’-O-MTase for capping the 5’ end of the viral RNA in association with viral GTase encoded by AmCPV S5.

**Expression and purification of recombinant p127**

The entire ORF of AmCPV S4 was expressed in *Escherichia coli* via a pQE-30 vector to produce a His-tagged fusion protein. The soluble protein was obtained by sonicating recombinant bacteria in Buffer A (see Methods) and purification using nickel-nitrilotriacetic acid (Ni-NTA)
chromatography. Analysis of purified protein by SDS-PAGE showed a single band of 127 kDa (Fig. 2a, lane 3) and indicated its purification to homogeneity. Purification of mutant (G831A) p127 also showed a single band in SDS-PAGE (Fig. 2a, lane 4).

Characteristics of the RTPase activity

In order to test whether p127 possessed RTPase activity, 277 bp of the 5'9 end of AmCPV S2 RNA was synthesized by in vitro transcription and its 5'-terminal nucleotide phosphate was labelled with 32P at the γ position. Incubation of this 5'-end-labelled RNA with p127 showed the release of labelled terminal phosphate as inorganic phosphate from substrate RNA (Fig. 2b, lane 4). A similar release of labelled terminal phosphate was observed when 5'-end-labelled RNA was incubated with purified virions (Fig. 2b, lane 5) or calf intestinal phosphatase (Fig. 2b, lane 3). Further analysis showed that the RTPase activity was dependent on the presence of divalent Mg2+ and the maximum activity was observed at 0.2 mM MgCl2 (Fig. 2c). To rule out the possibility of the release of terminal phosphate from contaminating Escherichia coli alkaline phosphatase, the assay of this enzyme was performed using eluted proteins from the Ni-NTA column of only vector containing induced bacterial lysate, but no activity was found (data not shown). The effect of temperature and pH on the RTPase was also analysed. The maximum activity of the RTPase and MTases of AmCPV p127 with different domains of other viral proteins. (a) Region of the putative 5' LxxR motif (shown in bold type and underlined) of VV (GenBank accession number AAA48253.1), WNV (NP_776018.1) and MRV (AAD42304.1). (b) Region of the putative GxGxG SAM-binding motif (shown in bold type and underlined) of WNV (GenBank accession number AF404756.1), JEV (AA21436.1) and MRV (Q91RA6.1). (c) Region of the putative N7-MTase (GDD) residues (shown in bold type and marked with an asterisk) of BmCPV VP3 [Protein Data Bank (PDB) ID: 3IZ3], Encephalitozoon cuniculi Ecm1 (PDB ID: 1Z3C_A) and VV (GenBank accession number AAA48253.1). (d) Region of the KDKE tetrad (shown in bold type and marked with an asterisk) of 2'-O MTase of BmCPV VP3 (PDB ID: 31Z3) and WNV (GenBank accession number NP_776018.1).
was observed at 37°C (Fig. 2d) and at pH 7.0 (Fig. 2e). From the kinetic analysis (Fig. 2f), $K_m$ and $V_{\text{max}}$ of this enzyme were calculated as 0.77 μM and 6.093 × 10$^{-14}$ mol min$^{-1}$, respectively, using the Michaelis–Menten equation.

**p127 has both N7- and 2'-O-MTase activity**

As it had been reported previously that AmCPV S5-encoded GTase and amino acid sequence alignment of AmCPV S4-encoded p127 showed the presence of a conserved SAM-binding motif, and a putative N7-MTase motif and 2'-O-MTase (KDKE) tetrad, an in vitro MTase assay was performed using 5'-end-labelled G*pppG-RNA and recombinant p127. The reaction products were fractionated through HPLC and the radioactivity of different peak fractions was determined by liquid scintillation counting. SAM, GpppG and m7GpppG were pre-equilibrated in the column as markers. Three major peaks for m7G*pppG, m7G*pppGm and as well as unreacted G*pppG were identified in HPLC fractions (Fig. 3b), but no such peaks for m7G*pppG or m7G*pppGm were observed when the same reaction was performed without SAM (Fig. 3a), indicating that p127 helped in the methylation of the 5'-cap structure, and SAM played a crucial role in N7 and 2'-O methylation in the newly synthesized RNA. These results also suggested that p127 not only possessed SAM-dependent N7-MTase activity, but also 2'-O-MTase activity. The site-directed mutagenesis at G831A in the SAM-binding domain did not show any peaks for m7G*pppG or m7G*pppGm, indicating that GxGxG played a critical role in the methylation of the 5'-cap structure of the viral RNA by transferring the methyl group from SAM (Fig. 3c).

**Optimization of guanine N7 and ribose 2'-O methylation by p127**

To determine the optimum pH, temperature and divalent cations for maximum MTase activity by p127, assays were performed at different pH, temperature and Mg$^{2+}$ concentration. The results showed that both the guanine N7- and ribose 2'-O-MTase activities reached a maximum when performed at 30°C (Fig. 4b), whereas the optimal pH and requirement for MgCl$_2$ differed. The optimum pH for N7 methylation was found to be pH 7.0 (Fig. 4a) and...
was inhibited by increasing concentrations of MgCl₂ (Fig. 4c). In contrast, the optimum pH for 2'⁹-O methylation was 10.0 (Fig. 4a) and activity was maintained at MgCl₂ concentrations from 2.55 to 10 mM (Fig. 4c). It has been reported that the N⁷- and 2'⁹-O-MTase of WNV, and guanine N²-MTase of mimivirus were inhibited by SAM analogues such as sinefungin or S-adenosy-l-homocysteine (SAH). To determine the effect of SAH on N⁷ methylation by AmCPV p127, a similar MTase reaction was performed in the presence of different concentrations of SAH. The results showed that SAH progressively inhibited the MTase of p127 in a concentration-dependent manner and completely inhibited activity at 500 μM (Fig. 4d).

**Sequential guanine N⁷ and ribose 2'⁹-O methylation of the RNA cap structure**

To determine the order of methylation by p127, a kinetic analysis was performed at different time points using G*pppG-RNA and m⁷G*pppG-RNA as substrate. Digestion of reaction products with nuclease P1 followed by TLC analysis and determination of radioactivity of different spots by liquid scintillation counting showed that when G*pppG-RNA was used as substrate, m⁷Gppp⁴G was first detected in 1 min, reached a maximum in 5 min and then steadily declined at later time points (~50% of G*pppG was converted into m⁷G*pppG at ~3 min) (Fig. 5a, b). When m⁷G*pppG-RNA was used as substrate, it was converted to m⁷G*pppGm at ~15 min (Fig. 5c, d). These results indicated that ribose 2'⁹-O methylation occurred only after the accumulation of the guanine N⁷-methylated cap and suggested a sequential methylation of the RNA cap in the order of GpppG > m⁷GpppG > m²GpppGm.

**Kinetic analysis of AmCPV N⁷-MTase**

To determine the kinetic parameters, enzyme analysis was performed either at a fixed concentration of RNA (2 μM) and varying concentrations of SAM (0–60 μM) (Fig. 6a) or at a fixed concentration of SAM (20 μM) and varying concentrations of RNA (0–10 μM) (Fig. 6b), and the standard kinetic parameters were calculated using the classical
Michaelis–Menten equation: 

\[ V_i = \frac{V_{\text{max}} [\text{SAM}]}{K_m + [\text{SAM}]} \]

where \( V_i \) is the initial velocity (pmol min\(^{-1}\)), \( V_{\text{max}} \) is the maximum velocity (pmol min\(^{-1}\)) and \( K_m \) is the substrate concentration (μM) at \( V_{\text{max}}/2 \). From this analysis, the \( K_m \) values for SAM and RNA were calculated as 4.41 and 0.39 μM, respectively. Similarly the \( V_{\text{max}} \) values for SAM and RNA were calculated as 0.88 and 0.94 pmol min\(^{-1}\), respectively.

**DISCUSSION**

We report here the cloning, sequencing, expression and functional characterization of genome S4 of *AmCPV*. S4 encodes a protein of 1110 aa with a deduced molecular mass of 127 kDa. The amino acid sequence alignment of this protein shows the presence of a RTPase domain (LRDR), a SAM-binding motif (GxGxG) and the KDKE tetrad of 2′-O-MTase, and suggests that it may participate in the capping of the 5′ end of nascent viral RNA. In several viruses, such as *BmCPV*, BTV and rotavirus, GTase and MTase activities are provided by one protein; however, in the insect orbivirus JKT-7400, these two enzyme activities are provided by two different proteins: VP4 provides MTase activity and VP6 provides GTase activity (Liao & Stollar, 1997a, b). In this respect *AmCPV* seems to be different from *BmCPV*, BTV and rotavirus, but similar to orbivirus, as it has been reported recently that *AmCPV* S5-encoded p65 provides GTase without any MTase activity (Biswas *et al.*, 2014). Therefore, for the completion of the capping reaction, MTase activity must be provided by proteins encoded by separate genome segments. Functional analysis of purified recombinant p127 expressed by *E. coli* shows that not only does it provide RTPase activity to remove γ-phosphate from the 5′ end of RNA to form a 5′-diphosphate-ended RNA, but it also helps to add a methyl group in the capped guanosine residue as well as in the first nucleotide of the RNA using SAM as the methyl group donor, confirming that S4-encoded p127 possesses both N7- and 2′-O-MTase activities, and together with S5-encoded p65 completes the capping reaction.

In the case of VV, WNV and MRV, the motif LRIR/LRPR/LKPR has been reported to be involved in RTPase activity (Bartlett & Joklik, 1988; Wengler & Wengler, 1993; Yu & Shuman, 1996). Amino acid residues 970–973 (LRDR) of p127 could be aligned properly with the RTPase motif of VV, WNV and MRV, indicating that active-site residues of *AmCPV* RTPase may also be located in these LRDR residues. The optimum temperature (37°C), pH (7.0) and Mg\(^{2+}\) (0.2 mM) requirement for *AmCPV* RTPase as well as the \( K_m \) (0.77 μM) and \( V_{\text{max}} \) (6.093 \times 10^{-14} \text{ mol min}^{-1})
are also comparable with those of MRV (optimum temperature 42°C, Mg²⁺ concentration 0.2 mM, $K_m$ 0.26 mM and $V_{max}$ $5.7 \times 10^{-14}$ mol min⁻¹⁻¹) (Bisaillon et al., 1997).

In the capping process, after the removal of the 5′ γ-phosphate of RNA by RTPase, transfer of GMP occurs to the 5′-diphosphate-ended RNA by GTase and then guanine N⁷ and 2′-O methylation occur with the help of MTases and SAM to complete the capping process (Issur et al., 2009). The results of our time-course analysis of MTase using $G^*_{pppG}$-RNA and $m^7G^*_{pppG}$-RNA show that 2′-O methylation occurs following $N^7$ methylation. The $K_m$ values of p127 ($N^7$-MTase) for SAM (4.41 μM) and RNA

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**Fig. 5.** (a–d) Time-course analysis of p127-mediated MTase activities. The *in vitro* transcribed 5′ UTR of S2 RNA with (a) $G^*_{pppG}$ or (c) $m^7G^*_{pppG}$ at the 5′ end was incubated with p127 for different time periods (as indicated) and analysed by TLC after digestion with nuclease P1. The conversion of $G^*_{pppG}$ to $m^7G^*_{pppG}$ and $m^7G^*_{pppG}$ to $m^7G^*_{pppG}m$ at each time point (determined through the measurement of radioactivity) is plotted in (b) and (d) [for experiments in (a) and (c), respectively].

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**Fig. 6.** Steady-state kinetic analysis of $N^7$ methylation by p127. Reaction velocity was determined by the incorporation of the methyl group in $m^7G^*_{pppG}$ in the 5′ UTR of AmCPV S2 RNA in (a) the presence of varying concentrations of SAM with constant amounts of RNA and (b) the presence of varying concentrations of RNA with constant amounts of SAM.
(0.39 μM) are comparable with those of VV N7 MTase (Chung et al., 2010). The lack of methylation in RNA in the absence of SAM or when using p127 with a mutant SAM-binding site (GLGVG) indicates that SAM plays a critical role in both N7 and 2'-O methylation. This has been further confirmed by the inhibition of both these methylation reactions by a SAM analogue, SAH, in a concentration-dependent manner, as has been reported for WNV and mimivirus (Benarroch et al., 2009; Dong et al., 2008; Martin & McMillan, 2002). A recent cryo-EM study of transcribing and non-transcribing BmCPV showed that its VP3 protein contains binding sites for both SAM and RNA in two MTase domains (MTase-1 and MTase-2) to help the 2'-O- and N7-MTase reactions (Zhu et al., 2014). A conserved catalytic tetrad KDKE is thought to be a characteristic of RNA 2'-O-MTase, within which Lys531, Lys649, and Glu689 serve to interact with the RNA sugar-phosphate backbone, and Asp616 plays a key role in SAM/SAH binding (Assenberg et al., 2007; Zhu et al., 2014). In addition to this conserved tetrad, Gly562, Asp589 and Val600 around the SAM/SAH structure of BmCPV VP3 have been predicted to be involved in 2'-O-MTase activity because the residues overlap closely with those in the 2'-O-MTase domain of BTV VP4 (Sutton et al., 2007), VV VP39 (Hodel et al., 1996) and coronavirus nsp16 (Decroly et al., 2011). In AmCPV p127, these KDKE sequence residues (Lys531, Asp561, Lys589 and Glu589) and other adjacent residues are also found to be present, indicating that these residues may interact with RNA and SAM for 2'-O-MTase activity. Residues 833–1058 of BmCPV VP3 (Gly563, Asp586 and Asp882) have been suggested to contain a N7-MTase domain as this region closely resembles Ecm1, a N7-MTase from Encephalitozoon cuniculi (Fabrega et al., 2004), and VP N7-MTase D1 (De la Peña et al., 2007; Zhu et al., 2014). In AmCPV p127, mutagenesis experiments showed the involvement of GLGVG domains in SAM binding, as has been reported for MRV (Martin & McMillan, 2002); however, involvement of additional residues such as Gly587, Gly592 and Asp508 (which could be aligned with Glu563, Asp586 and Asp882 of BmCPV VP3) is also possible. Further studies are required to identify the exact amino acid residues involved in the SAM-binding and N7-MTase activity of AmCPV p127. However, the molecular and biochemical characterization of AmCPV MTase may help to understand its role in viral replication and its use as a potential target for the development of antiviral compounds.

**METHODS**

cDNA synthesis, molecular cloning and sequencing of AmCPV S4. Purification of AmCPV polyhedra from virus-infected larvae was done following the method of Hayashi & Bird (1970) with some modifications (Qanungo et al., 2000). The total dsRNA genome was isolated from purified polyhedra by a modified guanidine isothiocyanate method (Chomczynski & Sacchi, 1995), separated by gel electrophoresis and S4 was eluted using a gel extraction kit (Qiagen). Conversion of the S4 dsRNA to its corresponding cDNA was done following the sequence-independent reverse transcription method of Andersen et al. (1992). The cDNA was cloned into pCR-XL-TOPO vector to create pCR-XL-TOPO/AmCPV S4, and after transforming into Escherichia coli TOP10 cells, plasmids were isolated and characterized by EcoRI restriction digestion. Recombinant plasmids containing the proper size insert were then sequenced by using BigDye in an automated DNA sequencer (ABI Prism 3100; Applied Biosystems) with M13 forward (5'-GGAAACACGTATGACCATG-3') and reverse (5'-AGCGGATAACAATTTACACAG-3') primers as well as internal primers designed from deduced sequences.

The sequence of AmCPV S4 was analysed by Sequencer (Gene Codes Corporation) and homology searches were done using BLAST (Altschul et al., 1997). The molecular mass, isoelectric point and amino acid composition were determined by ProtParam of ExPaSy (http://www.expasy.org/tools/protparam.html). Conserved motifs were identified using the Motif Scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and secondary structure was predicted using the GOR4 program (Garnier et al., 1996). Multiple sequence alignment was done by CLUSTAL_W to find conserved motifs among other related viruses and the similarity in amino acid sequences was defined using EScript 2.2 (Gouet et al., 1999).

**Expression and purification of p127.** The entire ORF of AmCPV S4 from nt 45 to 3375 (1110 aa) was amplified by PCR from plasmid pCR-XL-TOPO/AmCPV S4 using ACCUZYMÈ DNA polymerase (Bioline) and two synthetic primers AGCPV S4 F (5'-TATGGCATCA-3') and AGCPV S4 R (5'-TGGTCGCTTGACCCCTCTAGTT-3') containing Sadl (forward primer) and Sall (reverse primer) restriction sites (shown in bold type). The PCR product was digested with respective restriction enzymes and ligated to Sadl/Sall-digested pQE-30 vector to create pQE-30/AmCPV S4. Overlapping extension PCR-based site-directed mutagenesis (Ho et al., 1989) was performed to replace Gly831 with Ala (G831A) in the conserved GxGxG SAM-binding motif using primers AGCPV S4 GAF (5'-CCCTCGTGCTACCGTGTTGGCATAACA-3') and AGCPV S4 GAF (5'-TGTATGCGCCAAGGCGTACCGTTGGG-3'). The resulting recombinant plasmid was then transformed into Escherichia coli M15 cells, and colonies were screened by restriction digestion and confirmed by sequencing.

For the analysis of recombinant protein expression, Escherichia coli harbouring pQE-30/AmCPV S4 or pQE-30/AmCPV S4 G831A were grown in fresh 5 ml LB media at 37 °C to OD600 0.6. The culture was then induced with 1 mM IPTG for an additional 4 h at the same temperature. Bacterial cells were then harvested and analysed by 10 % SDS-PAGE (Laemmli, 1970).

To purify expressed protein (p127) in the soluble form, recombinant Escherichia coli harbouring pQE-30/AmCPV S4 were grown in 1 l LB media, induced with 1 mM IPTG, lysed by sonication in buffer A (10 mM Tris/HCL, pH 8.0, 300 mM NaCl and 1 % N-lauryl sarcosine) and recombinant protein was purified through Ni-NTA chromatography (Qiagen). The amount of purified protein was determined by the Bradford method (Bradford, 1976) using BSA as standard and the purity was checked by 10 % SDS-PAGE. The purified protein was dialysed overnight at 4 °C in 10 mM Tris/300 mM NaCl, pH 8.0, to remove the N-lauryl sarcosine. The protein was concentrated using a Centrificon YM-50 (Millipore) and used for functional analysis.

**Preparation of substrates for the RTPase assay.** To generate the 5' UTR of AmCPV S2 with pppG-ended RNA, *in vitro* transcription was performed using T7 (5'-GCTCTAGATTAATACGACTTATAGTTACGACCATGATCTATGACCATG-3') and T2 (5'-AATTCGCTAATTCGACTCACAC-3') primers and an *in vitro* transcription kit (Ambion). To perform the RTPase assay, 5'-end labelling of *in vitro* transcribed pppG-ended RNA (277 bp) was done using [γ-32P]ATP and enzyme according to the protocol of a RNA end-labelling kit (Ambion).
RTPase assay. The RTPase assay was performed as described by Wang et al. (2009). In brief, 1 μM γ-32P-labelled G-ended RNA (bp) was incubated with 1.5 μM p127 or virions (1 μg) in a buffer containing 250 mM Tris/HCl, pH 8.0, 100 mM NaCl and 1 mM MgCl2 (total volume 15 μl) at 37 °C for 1 h. Finally, the reaction was stopped by adding 0.1 M EDTA and aliquots (3 μl) were separated by TLC in a polyethyleneimine (PEI)-cellulose plate using 0.9 M LiCl buffer. The plate was dried, exposed to X-ray film and the radioactivity of separated spots was determined in a liquid scintillation counter (PerkinElmer). To determine the optimum reaction conditions, the RTPase assay was performed at different temperatures (15–50 °C), pH (4–9) and MgCl2 concentrations (0–1.5 mM).

The enzyme kinetic parameters were determined by incubating different concentrations of γ-32P-labelled RNA (0–10 μM) with 1.5 μM p127 in appropriate buffer as described earlier. Then the product was separated onto a PEI-cellulose plate in 0.9 M LiCl buffer followed by determination of radioactivity of separated spots in a liquid scintillation counter. Kinetic parameters such as apparent Km and apparent Vmax were determined by direct fit of rate versus substrate concentration data to the Michaelis–Menten equation (using GraphPad Prism 4.0 software) followed by a Lineweaver–Burk plot.

Preparation of substrates for the MTase assay. To perform the MTase assay, the terminal phosphate from the 5’ end of in vitro transcribed RNA was removed by incubating 1.5 μg RNA transcript with 1.5 μM p127 in a buffer containing 50 mM Tris/HCl, pH 7.9, 100 mM NaCl, 10 mM MgCl2 and 1 mM DTT at 37 °C for 1 h. Finally, 5’-diphosphate-ended RNA was extracted with phenol/ chloroform (1:1, v/v), precipitated with ethanol and resuspended in nuclease-free water. For capping, 1 μg 5’-diphosphate-ended RNA transcripts (ppG-RNA) was incubated in a buffer containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 5 mM DTT, 30 μM [z-32P]GTP and 5 μg S5-encoded recombinant p65 (GTase) at 37 °C for 30 min to transfer the GMP moiety to the 5’-diphosphate-ended RNA creating G’pppG-RNA (Biswas et al., 2014). RNA was extracted with phenol/ chloroform (1:1, v/v), precipitated with ethanol and resuspended in DEPC-treated water.

MTase assays. The N2-MTase assay was performed by incubating 2 μM G’pppG-RNA with 20 μM SAM and 1.5 μM p127, in a reaction buffer containing 50 mM Tris/HCl, pH 7.5, 6 mM DTT and 2 mM MgCl2 at 30 °C for 5 min. For the 2’-O-MTase assay, the same experiment was performed for a longer incubation period (30 min) at 30 °C. At the end of the incubation, the RNA was extracted by phenol/ chloroform (1:1, v/v) extraction and precipitated with ethanol. The RNA was then digested by nuclease P1 (US Biological), and the reaction products were separated by HPLC using an anion-exchange RNA was then digested by nuclease P1 (US Biological), and the reaction products were separated by HPLC using an anion-exchange TLC in a PEI-cellulose plate and after autoradiography the radioactivity of each separated spot was determined in a liquid scintillation counter. To determine the effect of SAH, MTase assays were performed by adding varying concentrations of SAH (0–1000 μM) in the same reaction for 30 min at 30 °C.

Time-course analysis of N2 and 2’-O methylation by p127. For time-course analysis of the N2 methylation reactions, 2 μM G’pppG-RNA was incubated with 1.5 μM p127 and 20 μM SAM for different time periods (0–30 min). The reaction products were digested with nuclease P1 and then one half of the reaction products was analysed on a PEI-cellulose TLC plate and autoradiographed. Radioactivity of each of the separated spots was then measured in a scintillation counter. The rest of the nuclease P1-digested reaction products obtained from the 5 min time period were extracted by phenol/ chloroform (1:1, v/v), precipitated by ethanol and analysed by HPLC (as described earlier). Fractions containing m7G’pppG were used as substrate for the ribose 2’-O methylation reaction by incubating with 20 μM SAM and 1.5 μM p127 for 0–30 min. and analysis on PEI-cellulose TLC plates (Dong et al., 2008; Ray et al., 2006).

Kinetic analysis. To determine Km for SAM during N2-MTase reactions by p127, an assay was carried out using 2 μM G’pppG-RNA, 1.5 μM p127 and varying concentrations of SAM (0–60 μM) (Dong et al., 2008). Similarly, Km for RNA was determined by varying the concentration of G’pppG-RNA (0–10 μM) in the reaction and keeping SAM at 20 μM at 37 °C for 5 min. The reaction products were then analysed by TLC. The kinetic parameters were obtained by fitting the experimental data to GraphPad Prism v5.

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