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Characterization of the guanine-N7 methyltransferase activity of coronavirus nsp14 on nucleotide GTP

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ABSTRACT

Most eukaryotic viruses that replicate in the cytoplasm, including coronaviruses, have evolved strategies to cap their RNAs. In our previous work, the nonstructural protein (nsp) 14 of severe acute respiratory syndrome coronavirus (SARS-CoV) was identified as a cap (guanine-N7)-methyltransferase (N7-MTase). In this study, we found that GTP, dGTP as well as cap analogs GpppG, GpppA and m7GpppG could be methylated by SARS-CoV nsp14. In contrast, the nsp14 could not modify ATP, CTP, UTP, dATP, dCTP, dUTP or cap analog m7GpppA. Critical residues of nsp14 essential for the methyltransferase activity on GTP were identified, which include F73, R84, W86, R310, D331, G333, P335, Y368, C414, and C416. We further showed that the methyltransferase activity of GTP was universal for nsp14 of other coronaviruses. Moreover, the accumulation of m7GTP or presence of protein nsp14 could interfere with protein translation of cellular mRNAs. Altogether, the results revealed a new enzymatic activity of coronavirus nsp14.

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1. Introduction

Eukaryotic and most viral mRNAs possess a 5'-terminal cap structure, m7G(5)ppp(5)N-, which serves as a basic recognition site in translation and is important for efficient splicing, nuclear export, translation and stability of mRNA against the attack of 5'exonucleases in eukaryotic cells (Cougot et al., 2004; Furuichi and Shatkin, 2000; Schwer et al., 1998). In eukaryotic nucleus, the formation of the cap structure needs a series of enzymatic activities (Shuman, 1995). First, the γ -phosphate at the 5'-end of nascent mRNA is removed by RNA triphosphatase (TPase). Second, the GMP moiety derived from a covalent enzyme-GMP intermediate is transferred to the diphosphate mRNA via a two-step reaction by guanylyltransferase (GTase). Finally, the GpppN cap is methylated by S-adenosyl-L-methionine (AdoMet or SAM)-dependent RNA (guanine-N7) methyltransferase (N7-MTase) at position 7 of the terminal guanosine. Although the final cap structures of viral and cellular mRNAs are very similar, RNA viruses have evolved

diversified mechanisms to cap their mRNAs that are thus translated in the manner of eukaryotic mRNAs (Lai et al., 1982; Martin et al., 1975; Sagripanti et al., 1986; Shuman et al., 1980). For example, vaccinia virus employs a canonical pathway for mRNA capping, and the cap-0 structure at the 5'-end of vaccinia virus mRNA is formed by D1 (containing RNA TPase and GTase) and D12 (N7-MTase) by a mechanism analogous to the nuclear functions. Alphaviruses employ a non-canonical pathway, in which GTP is converted into m7GTP by viral N7-MTase before being transferred to RNA by GTase (Ahola and Ahlquist, 1999; Ahola and Kaariainen, 1995; Huang et al., 2005).

The family *Coronaviridae*, comprising the subfamily *Coronavirinae* and *Torovirinae*, belongs to the order *Nidovirales*, a lineage of positive-strand RNA viruses that also includes the *Roniviridae*, *Arteriviridae* and *Mesoniviridae* families (Gorbalenya et al., 2006; Lauber et al., 2012). Coronaviruses are frequently associated with respiratory and enteric diseases in humans, livestock, and companion animals. On the basis of immunogenicity and molecular evolutionary relationship, coronaviruses have been divided into three groups: group 1 is exemplified by human coronavirus 229E (HCoV-229E) and NL63, the porcine transmissible gastroenteritis virus (TGEV), and feline coronavirus (FCoV), group 2 includes SARS coronavirus (SARS-CoV) which causes the life-threatening severe acute respiratory syndrome (SARS), murine hepatitis virus (MHV), the human coronavirus (HCoV-OC43) and HKU1, and group 3 includes infectious bronchitis virus (IBV). Coronaviruses possess a nearly 30 kb positive-stranded RNA genome, and the two large open reading frames (ORFs) 1a and 1b, located at the 5'-two-thirds of

Abbreviations: SARS-CoV, severe acute respiratory syndrome coronavirus; nsp, nonstructural protein; MTase, methyltransferase; AdoMet, S-adenosyl-L-methionine; RTC, replication and transcription complex.

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the genome, encode the proteins making the replication and transcription complex (RTC). Previous studies have shown that nsp13 functions as RNA helicase and 5'-triphosphatase (Ivanov et al., 2004; Tanner et al., 2003), and we and others identified nsp14 as an exoribonuclease and N7-MTase (Chen et al., 2007, 2009; Minskaia et al., 2006), and nsp16 as a 2'-O methyltransferase (2'-O-MTase) (Chen et al., 2011; Decroly et al., 2008). Currently the GTase of coronavirus is still unknown.

Eukaryotic initiation factor 4E (eIF4E) is a component of the heterotrimeric complex eIF4F and has central roles in the control of several aspects of gene expression at the post-transcriptional level. Traditionally, eIF4E plays a major role in cap-dependent translation initiation where it binds the 5'-m7G cap found on mRNAs (Culjkovic et al., 2007; Gingras et al., 1999; von der Haar et al., 2004). Free m7GTP was known as a potent inhibitor of cap-dependent translation *in vitro* by competing with mRNA for eIF4E (Cai et al., 1999; von der Haar et al., 2004). It was reported that alphavirus capping enzyme can methylate GTP and dGTP, as well as 5'-5' dinucleotides containing guanosine, and the vaccinia virus N7-MTase can also catalyze, but less efficiently, methylation of GTP and dGTP (Ahola and Ahlquist, 1999; Laakkonen et al., 1994; Martin and Moss, 1976; Scheidel et al., 1989). However, there is no direct evidence that these viral capping enzymes can inhibit translation by GTP methylation.

In our previous studies, we showed that SARS-CoV nsp14 could methylate the cap structure of different substrate RNAs that possessed a GpppG or GpppA cap (Chen et al., 2009). In this study, we found that coronavirus nsp14 could also utilize GTP and dGTP as well as cap analogs GpppG, GpppA and m7GpppG as substrate for methylation. Furthermore, we performed systematic mutagenesis of SARS-CoV nsp14 and identified the critical amino acid residues essential for GTP methylation activity of SARS-CoV nsp14.

2. Materials and methods

2.1. Construction of plasmids

The coding sequences for nonstructural proteins (nsp1–16) of SARS-CoV, nsp14 of TGEV and nsp 14 of MHV, were PCR amplified from cDNAs of SARS-CoV strain WHU, TGEV, MHV, and inserted into the protein expression vector pET30a (Novagen). The mutants of nsp14 of SARS-CoV were generated by overlap PCR with mutagenic primers from SARS-CoV strain WHU, and the PCR fragments were cloned into plasmid pET30a. For all eukaryotic expression plasmids, SARS-CoV nsp14, D331A and nsp16, the corresponding sequences were amplified from protein expression plasmids by PCR and sub-cloned into the eukaryotic expression vector pRK-flag. All of the clones were confirmed by DNA sequencing.

2.2. Cell culture and DNA transfection

293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. All transfection was performed using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer.

2.3. Protein expression and purification

All recombinant protein expression plasmids except nsp12 were transformed into *Escherichia coli* BL21 (DE3) cells. Cultures were grown in Luria-Bertani (LB) medium containing kanamycin (50 µg/ml) at 37 °C and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 16 °C for 12–16 h. Then the cells were collected by centrifugation and resuspended in buffer A

[50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgSO₄, 5% glycerol] supplemented with 10 mM imidazole. After cell lysis by sonication, the cell lysate was separated by centrifugation at 24,000 × g for 20 min, and the filtrated supernatant was applied to nickel-nitrilotriacetic acid (Ni-NTA) resin (Genescript) and washed with buffer A supplemented with an imidazole gradient of 20 mM, 50 mM, and 80 mM. Protein was eluted with buffer A supplemented with 250 mM imidazole. At last, the elution buffer was changed to reaction buffer [50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM DTT, 10% glycerol] and the fractions were frozen at –80 °C. The purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Chen et al., 2007). The expression and purification of recombinant SARS-CoV nsp12 were described previously (te Velthuis et al., 2010).

2.4. Biochemical assays for MTase activity

The MTase activity assays with ³²P-labeling were carried out in 10 µl reaction mixture [2 µM of purified recombinant proteins, 0.3 pM of ³²P-labeled GTP, 2 mM of GTP, 40 mM Tris-HCl (pH 7.5 or 8.0), 2 mM MgCl₂, 2 mM DTT, 10 units RNase inhibitor, 0.2 mM AdoMet] and incubated at 37 °C for 1.5 h, then spotted onto polyethyleneimine cellulose-F plates (Merck) for thin layer chromatography (TLC), and developed in 0.4 M ammonium sulfate. The extent of ³²P-labeled cap was determined by scanning the chromatogram with a PhosphorImager as described previously (Chen et al., 2009).

The MTase activity assays with ³H-labeling were carried out in 30 µl reaction mixture [40 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 2 mM DTT, 40 units RNase inhibitor, 0.01 mM AdoMet], with 14.9 pM of Ado[methyl-³H]Met (67.3 Ci/mmol, 0.5 µCi/µl), 4 µM of purified proteins, and 2 mM of NTPs or other RNA substrates (m7GpppA/GpppA/m7GpppG/GpppG cap analog) at 37 °C for 1.5 h. ³H-labeled product was isolated in small DEAE-Sephadex columns and quantitated by liquid scintillation (Ahola et al., 1997). The reaction mixtures were also analyzed by 12% SDS-PAGE. The gels were soaked in Enlightening buffer (Perkin-Elmer) and dried under vacuum with heat at temperature below 95 °C. The dried gels were placed against a suitable X-ray film and exposed at –80 °C, until the desired visualization level is achieved (Ahola et al., 1997).

2.5. Covalent guanylate binding assays

Covalent guanylate binding reactions were performed in a 30-µl final volume with 50 mM HEPES (pH 7.2), 10 mM KCl, 2 mM MgCl₂, 5 mM dithiothreitol, 1.2% n-octyl-β-D-glucopyranoside, 100 µM AdoMet, and 2 µCi of [α-³²P]GTP. The reaction mixtures were incubated for 20 min at 30 °C, and reactions were stopped by addition of sodium dodecyl sulfate (SDS) to 2% (final concentration) followed by boiling for 3 min. The samples were analyzed by SDS-PAGE and visualized by scanning the chromatogram with a PhosphorImager (Ahola and Ahlquist, 1999).

2.6. *In vitro* translation assay

Recombinant protein was incubated with 10 µl of rabbit reticulocyte lysate (RRL, Promega), and after 1 h, 200 ng of firefly luciferase-encoding mRNA and amino acids were added to the mixture and incubated for another 1.5 h at 30 °C, followed by a luciferase activity assay. In addition, indicated concentration of m7GTP was added to the preincubation mixtures of 10 µl of RRL and subjected to an *in vitro* translation system with 200 ng of luciferase mRNA. Luciferase activity was measured with a TD-20/20 spectrophotometer (Promega).

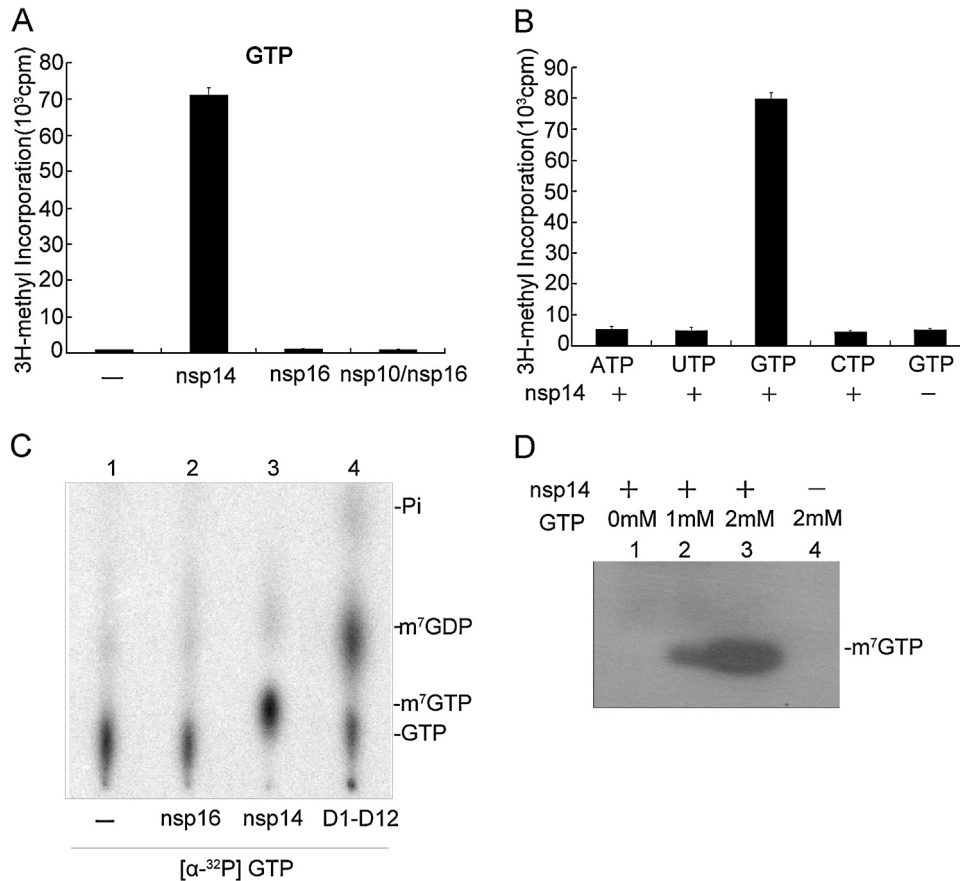


Fig. 1. Methylation of GTP by SARS-CoV nsp14. (A) The GTP methylation was carried out in a 30- μ l solution that contained purified recombinant protein, Ado[methyl-³H]Met, and GTP. Radioactivity incorporated into the substrate was measured by liquid scintillation counting, and the average counts per minute for each condition is displayed by the histogram bars. Standard deviations are indicated by error bars. (B) The substrates indicated below the bars were assayed as methyl group acceptors in standard reactions with nsp14. The error bars indicate standard deviation. (C) The GTP methylation reaction solution contained the indicated protein, AdoMet, and [α -³²P]GTP. The radiolabeled products generated in the reaction were analyzed by TLC followed by autoradiography. Vaccinia D1–D12 was used as a control. (D) The GTP methylation was carried out in the presence of Ado[methyl-³H]Met as described in (A), and non-labeled GTP was used at concentrations of 0, 1, and 2 mM. Bovine serum albumin was used as negative control (lane 4). The samples were analyzed by SDS-PAGE and autoradiography.

3. Results

3.1. SARS-CoV nsp14 catalyzes the transfer of methyl group to GTP

Our previous study has shown that SARS-CoV nsp14 acts as N7-MTase and can methylate RNA cap of both GpppA-RNA and GpppG-RNA. In this study, we attempted to test whether SARS-CoV nsp14 N7-MTase could also utilize single nucleotides as substrates for methylation. We first tested for the ability of nsp14 to methylate GTP, using radioactive Ado[methyl-³H]Met as the methyl donor. As shown in Fig. 1A, nsp14 showed robust methyltransferase activity on substrate GTP at a level 70 times above background (column 2), indicating that SARS-CoV nsp14 could also use free GTP as methylation substrate. SARS nsp16 alone or the nsp16/nsp10 complex, which acts as 2'-O-methyltransferase but cannot methylate GTP, were used as negative control (Fig. 1A, columns 3 and 4). We further tested whether SARS-CoV nsp14 could also methylate other nucleotides. As shown in Fig. 1B, nsp14 was not capable to methylate ATP, CTP, or UTP (Fig. 1B).

To confirm the chemical modification of GTP by nsp14 N7-MTase, we performed the methylation reaction with radioactive [α -³²P]GTP as substrate and analyzed the product by thin-layer chromatography (TLC). Our previous work showed that m7GTP could be readily separated from non-methylated GTP by TLC. As

shown in Fig. 1C, GTP was efficiently transformed into m7GTP by SARS nsp14 but not by the negative control nsp16. Vaccinia virus capping enzyme D1/D12, which contains RNA TPase and GTase in D1 and N7-MTase in D12, was used as positive control. The N7-MTase possesses weak activity of GTP methylation and the methylated GTP (m7GTP) is transformed into m7GDP by the intrinsic TPase, therefore, GTP methylation by vaccinia D1/D12 was not efficient and the final product was m7GDP instead of m7GTP (Fig. 1C, lane 4).

To affirm GTP specificity of the methyl transfer reaction catalyzed by nsp14, the methylation product was also analyzed in SDS-PAGE. As shown in Fig. 1D, the signal intensity of radioactive ³H was GTP concentration-dependent (Fig. 1D, lanes 1–3). Control protein (BSA) was not able to catalyze the methyl transfer into the substrate GTP (Fig. 1D, lane 4). All together, these data suggested that SARS nsp14 could use free GTP as methylation substrate and efficiently transform GTP into m7GTP.

3.2. Deoxy GTP and dinucleotide cap analogs can also be methylated by SARS-CoV nsp14

We further tested whether deoxy nucleotides and dinucleotide cap analogs could be methylated by SARS-CoV nsp14. As shown in Fig. 2A, dGTP could also be used as a methyl acceptor substrate by nsp14, while nsp14 showed no methylation

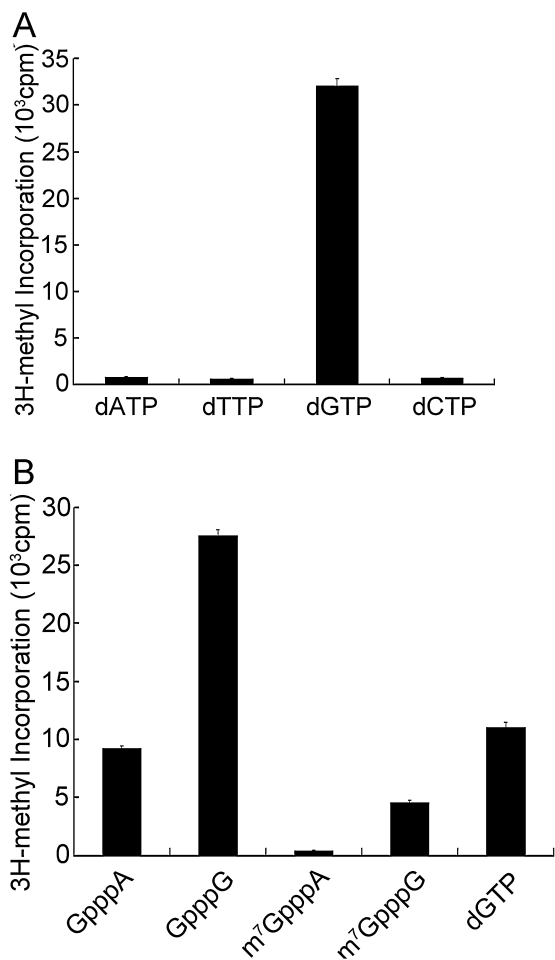


Fig. 2. Methylation of dGTP and dinucleotide cap analogs by SARS-CoV nsp14. (A) Different deoxynucleotides were used to test the methylation activities of nsp14 of SARS-CoV. Standard deviations are indicated by error bars. (B) The dinucleotide cap analogs indicated below the bars were assayed as methyl group acceptors in standard reactions with SARS-CoV nsp14. The error bars indicate standard deviation.

activity on dATP, dCTP, or dTTP (Fig. 2A). Then we observed that the dinucleotide cap analogs GpppG, GpppA and m7GpppG could be efficiently methylated by nsp14 as expected (Fig. 2B). However, the cap analog m7GpppA, which does not contain a free guanine-N7 position, could not be further methylated by nsp14 (Fig. 2B, column 3), indicating that nsp14 methylation was specific for position N7 of guanosine. Moreover, nsp14 also exhibited some activity toward m7GpppG, which has one non-methylated guanosine (Fig. 2B, column 4). However, this was less than 20% of the activity toward GpppG, suggesting that the 7-methylated guanosine already present in m7GpppG has an inhibitory effect on nsp14 MTase. The activity toward GpppG was about two fold of GpppA, as GpppG has a symmetrical structure with two N7 positions for accepting methyl groups. Furthermore, we also tested whether internal guanylyl residues in RNA could be adopted as acceptors for methyl groups, but no methylation activity of nsp14 could be detected (data not shown). Taken together, these results showed that SARS-CoV nsp14 could methylate guanosine with three phosphates at N7-position in either oxy or deoxy form.

3.3. N7-MTase activity on GTP is universal for nsp14 of other coronaviruses

We further tested whether the nsp14 of other coronaviruses also have the ability of GTP methylation. Therefore, we expressed and

purified MHV nsp14 (Fig. 3A) and TGEV nsp14 (Fig. 3B), and tested in biochemical MTase assays. As shown in Fig. 3C, nsp14 of MHV was able to methylate GTP and dGTP as efficiently as SARS-CoV nsp14 did. Nsp14 of TGEV, a member of group 1 coronaviruses that are distantly related to SARS-CoV, could also catalyze the transfer of methyl group to GTP and dGTP (Fig. 3D). These data indicate that N7 methylation activity on free GTP and dGTP are universal property for coronaviruses.

3.4. No covalent binding of guanylate with SARS-CoV proteins could be detected

As the GTase has not been identified for coronaviruses, we could not test whether the m7GTP can be transferred onto RNA substrate. In members of the alphavirus-like superfamily, the capping enzyme catalyzes GTP methylation and the following transfer of m7GMP from the newly formed m7GTP to RNA via a covalent [enzyme-m7GMP] intermediate (Ahola and Ahlquist, 1999). Formation of m7GTP by SARS nsp14 raised the question whether m7GTP could subsequently provide its m7GMP moiety to form the covalent [enzyme-m7GMP] intermediate. To answer this question, we expressed and purified the major SARS-CoV nonstructural proteins and used radioactive [α -³²P]GTP for methylation and binding assays. The vaccinia virus capping enzyme D1–D12 was used as a positive control. The protein products were analyzed by SDS-PAGE and visualized by fluorography (Fig. 4). The results showed that D1–D12 could support the formation of the covalent [enzyme-GMP] intermediate, but neither m7GTP nor GTP could covalently bind with the SARS-CoV nonstructural proteins tested.

3.5. Identification of critical amino acid residues of SARS-CoV nsp14 involved in GTP methylation activity

Our previous study showed that the core domain of the SARS-CoV nsp14 N7-MTase is located at the C-terminal half but the ExoN domain at the N-terminal half is also important for the N7-MTase activity of nsp14 (Chen et al., 2009). To further test the direct role of nsp14 in GTP methylation and to gain insight into the amino acid residues of nsp14 critically involved in GTP methylation, we chose 21 residues of nsp14 which are conserved among coronaviruses and generated site-directed mutants as described in our recent work (Chen et al., 2013). The 23 SARS-CoV nsp14 mutants were expressed in *E. coli* cells and purified by nickel–nitrilotriacetic acid affinity chromatography for assaying the N7-MTase activity on GTP. The ³H-labeled reaction products were purified and quantified by liquid scintillation. As shown in Fig. 5, The mutations F73A, R84A, W86A, R310A, D331A, D331Y, G333A, P335A, Y368A, C414R and C416R abolished the N7-MTase activity on GTP (Fig. 5, lanes 2–4, 7, 8, 10, 12, 17, 20, 21), while substitution of Ala for I332, K336, D352, Y420 and T428, and mutations C382Y and L419R severely weakened the GTP methylation activity to 15–50% in comparison with that of wildtype nsp14 (Fig. 5, lanes 11, 15, 16, 18, 22–25). The rest of the substitutions had marginal effects, with activities in the range of about 80% of that of wildtype nsp14. Among the ten critical residues identified, seven are located in the C-terminal MTase domain, and three of them (F73, R84 and W86) are located in the N-terminal ExoN domain of nsp14. These critical residues are the same as that identified for N7-MTase activity on RNA cap structure reported recently by our group (Chen et al., 2013), excepting that F73A mutation did not apparently influence the MTase activity on RNA cap. These results indicate that the ExoN domain is also needed for GTP methylation activity, which is consistent with our previous results (Chen et al., 2009).

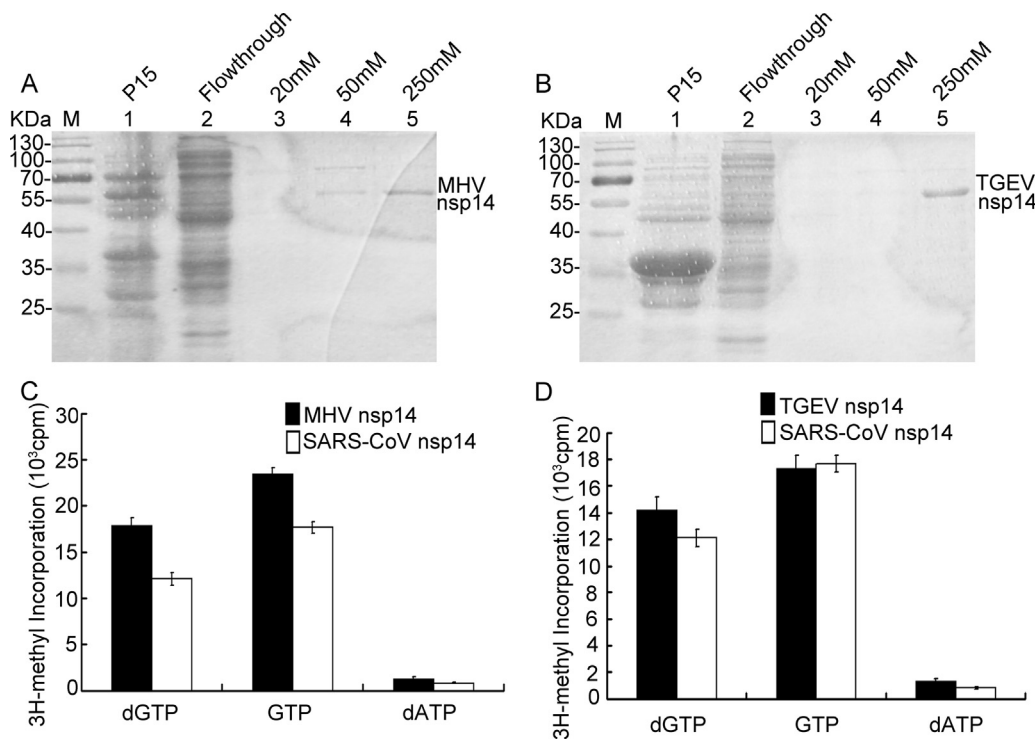


Fig. 3. Methylation of GTP and dGTP by MHV and TGEV nsp14. (A) and (B) MHV and TGEV nsp14 were purified and analyzed by SDS-PAGE. The sizes of protein markers are indicated on the left. (C) and (D) The methyltransfer reaction solution contained the MHV nsp14 (C) or TGEV nsp14 (D), Ado[methyl-³H]Met, and GTP or dGTP as described in Section 2. SARS-CoV nsp14 was used as a positive control. Radioactivity incorporated into the substrate was measured by scintillation counting. The error bars indicate standard deviation.

3.6. SARS-CoV nsp14 and its methylation product m7GTP can inhibit protein translation

It is known that the eukaryotic cellular mRNA N7-MTase involved in RNA capping is not active toward GTP and m7GTP can inhibit cap-dependent translation by competing with mRNA for eIF4E (Cai et al., 1999). To answer the question of whether the GTP methylation catalyzed by SARS-CoV nsp14 can influence cap-dependent protein translation, we first performed the translation efficiency assay in rabbit reticulocyte lysate (RRL) with in vitro transcribed and capped RNA containing β -globin 5'UTR and the coding region for firefly luciferase to allow determination of protein synthesis by luminometry. As shown in Fig. 6A, m7GTP could markedly inhibit the translation in a dose-dependent manner, and the inhibitory concentration of 50% was approximately 15 μ M. These data suggest that m7GTP is a potent inhibitor of cap-dependent translation, which is consistent with previous report (Cai et al., 1999). We then added directly the recombinant SARS-CoV nsp14 (instead of m7GTP) into the in vitro translation mixture, and a 50% inhibition of luciferase translation was observed by the addition of a final concentration of 0.2 μ M of nsp14, while 99% inhibition was obtained at 1 μ M. Thus, 0.2 μ M of nsp14 had equivalent inhibitory effect to that of 15 μ M m7GTP. When nsp14 D331A, which is defective in GTP methylation activity and did not reduce ExoN activity of nsp14 in the absence of nsp10 (Chen et al., 2009, 2013), was added into the translation system, translation was not apparently affected (Fig. 6B), indicating that the N7-MTase activity was responsible for the inhibition. These data suggest that nsp14 may have transformed GTP into m7GTP in the translation system and the resulting m7GTP may compete with mRNA cap for eIF4E and consequently inhibit mRNA translation.

To assess the possible effect of SARS-CoV nsp14 on translation in cells, 293T cells were co-transfected with a luciferase reporter plasmid together with nsp14 expression plasmid or controls. To exclude

the inhibitory effects of nsp14 on transcriptional regulation, we used two Renilla luciferase reporter plasmids, pRL-TK and pRL-SV40, which carry different promoters. As shown in Fig. 6C, a more than 80% inhibition of luciferase (TK) was observed by transfection of 200 ng of nsp14-expressing plasmid, while about 99% inhibition was achieved with 500 ng. In contrast, nsp16 did not inhibit the translation in the same test system. It was reported that SARS-CoV N protein could inhibit protein translation, therefore we also used N protein as a control, but we only could observe inhibition at low dosage (Fig. 6C). When the reporter pRL-SV40 was used, the similar inhibitory effects by nsp14 were observed (Fig. 6D). To further assess the effect of nsp14 on translation in cells, 293T cells were co-transfected with a luciferase reporter plasmid and pRK-nsp14 or pRK-D331A. As expected, the D331A mutant did not significantly inhibit the reporter activity (Fig. 6E). These results imply that the GTP methylation activity of nsp14 might play a role in regulating the protein translation of host cell mRNAs, which will be discussed in the next section.

4. Discussion

Coronaviruses have a nearly 30 kb positive-stranded genome and employ a complicate replication and transcription complex (RTC) for genome replication and expression. The RTC consists of 16 viral nonstructural proteins, and the enzymatic activities postulated to be involved in the RNA capping pathway were previously documented for nsp13 as RNA 5'-TPase/helicase (Ivanov et al., 2004; Tanner et al., 2003), nsp14 as N7-MTase and ExoN (Chen et al., 2007, 2009; Minskaia et al., 2006), and nsp16 as 2'-O-MTase (Chen et al., 2011; Decroly et al., 2008). In our previous study, SARS nsp14 was shown to be able to methylate the cap structure of GppRNA, and the coronavirus cap structure methylation appears to follow the canonical sequence of guanylyl transfer preceding the N7-methylation (Chen et al., 2009). In this report, we extended our

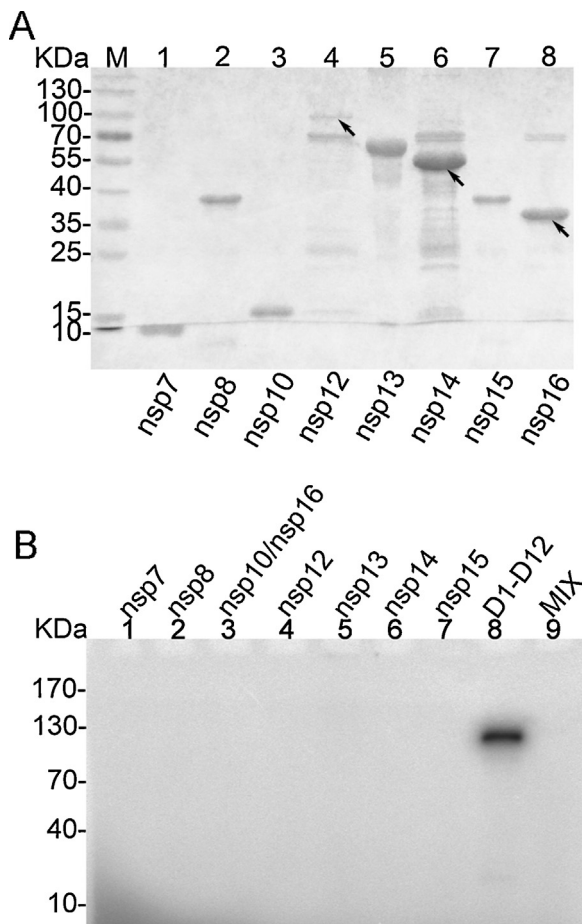


Fig. 4. Assays for covalent binding of guanylate with viral proteins. (A) Purification and SDS-PAGE analysis of recombinant proteins (lanes 2–8). The sizes of protein markers (lane 1) are indicated on the left. (B) The reaction solution contained SARS-CoV nonstructural protein, AdoMet, and [α - 32 P]GTP as described in Section 2 (lane 1–7). The protein mix included nsp7, nsp8, nsp10 and nsp12–16 (lane 9). Vaccinia D1–D12 was used as a positive control (lane 8). Formation of the protein-GMP intermediate was detected on a 10% SDS-PAGE gel by fluorography.

previous studies and found that coronavirus nsp14 also possess the N7-MTase activity on free GTP. This raises the question whether coronaviruses could use m7GTP for guanylyl transfer onto RNA as that of alphaviruses (Ahola and Kaariainen, 1995). However, the GTase for coronaviruses is still unknown, and the order of N7-methylation and RNA guanylyl transfer cannot be approved yet.

In this study, we showed that nsp14 could methylate GTP, dGTP as well as cap analogs GpppG, GpppA and m7GpppG, indicating that

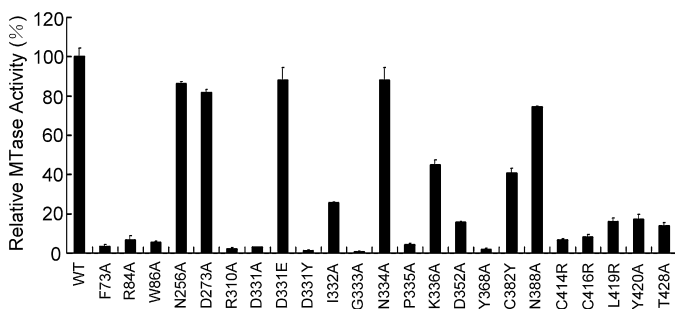


Fig. 5. Effects of mutations on the GTP methylation activity. The methyltransferase reaction solution contained the indicated protein, Ado[methyl- 3 H]Met, and GTP as described in Section 2. The activity of wildtype nsp14 is designated as 1. The error bars indicate standard deviation.

nsp14 specifically targets the guanosine. Because the m7GpppA is already fully methylated at the N7 position and cannot accept additional methyl groups, nsp14 was not able to methylate m7GpppA, indicating that methylation took place at the N7 position of the guanine ring. The nsp14 of TGEV and MHV were able to methylate GTP and dGTP efficiently as SARS-CoV nsp14 did, suggesting that GTP methylation function of nsp14 may be conserved among coronaviruses. Alphavirus SFV nsp1 can methylate GTP, dGTP and GpppG, but not GpppA, or in vitro transcribed RNAs with GpppA and GpppG caps (Ahola and Ahlquist, 1999; Laakkonen et al., 1994). The vaccinia virus N7-MTase can also catalyze, but less efficiently, methylation of GTP, dGTP and dinucleoside triphosphates with the structure G(5')pppN (Martin and Moss, 1976). Eukaryotic cellular N7-MTase catalyzes the methylation of polyribonucleotides with GpppN-sequences, and of the dinucleoside triphosphate G(5')pppG but not of GTP (Ahola and Ahlquist, 1999; Laakkonen et al., 1994; Martin and Moss, 1976; Scheidel et al., 1989). West Nile virus (WNV) is a member of the genus *Flavivirus*, and the cap methylation by WNV NS5 was sequence-specific for viral RNA (Dong et al., 2007). In summary, the properties of viral N7-MTases vary among different virus groups, and the enzymatic properties displayed by coronaviruses nsp14 and alphavirus capping enzyme share some similarities. This may imply the possibility that coronaviruses employ a non-canonical pathway for mRNA capping.

By the analysis of the mutants of SARS-CoV nsp14, we demonstrated that ten critical residues of nsp14 are essential for full activity of GTP methylation (Fig. 4). In our recent study, we systematically identified the critical residues of nsp14 for N7-MTase activity on capped GpppA-RNA (Chen et al., 2013). In comparison, 9 of the 10 critical residues essential for MTase activity on free GTP are also essential for the methylation of capped RNA. The residue F73 of nsp14 was not essential for the methylation of capped RNA but critical for GTP methylation. Why the residue F73 is closely involved in N7 methylation of free GTP but not capped RNA is not clear. Resolution of the crystal structure of nsp14 is needed to answer this question.

In previous studies, m7GTP and other cap analogs were shown to inhibit cap-dependent translation in vitro by competing with mRNA for eIF4E, and this was confirmed in this study. In this report, we showed that SARS-CoV nsp14 but not the MTase-defective D331A mutant could inhibit the in vitro translation similarly as m7GTP (Fig. 6), suggesting that nsp14 could efficiently transform GTP into m7GTP in the translation system and consequently inhibit the mRNA translation. In the nsp14 MTase activity assays in this study, the GTP concentration in the reaction was 2 mM, similar to the endogenous level of GTP in eukaryotic cells (Ditzelmüller et al., 1983). Efficient transformation of GTP into m7GTP in this system may suggest that nsp14 could work effectively at physiological condition. We also showed that expression of nsp14 but not the MTase-defective D331A mutant led to the inhibition of luciferase reporter activity in cells. However, the test system adopted was either the in vitro biochemical assays or transient over-expression of nsp14 in cells. Therefore, it is still unclear whether coronavirus nsp14 could inhibit mRNA translation in host cells during virus infection. It was reported that SARS-CoV nsp1 could inhibit host protein synthesis and promote host mRNA degradation, but nsp1-defective SARS-CoV mutant could still inhibit host protein synthesis (Kamitani et al., 2006; Narayanan et al., 2008), suggesting that SARS-CoV may possess other mechanisms to suppress protein translation in infected cells. It has been reported that intracellular levels of GTP are in the millimolar range in eukaryotic cells (von der Haar et al., 2004) and therefore there is opportunity for nsp14 to methylate GTP in cells. Further studies are needed to address the in vivo functions of nsp14 related to protein translation.

In summary, we provided direct evidence that coronavirus nsp14 could methylate free GTP. These results confirm and extend

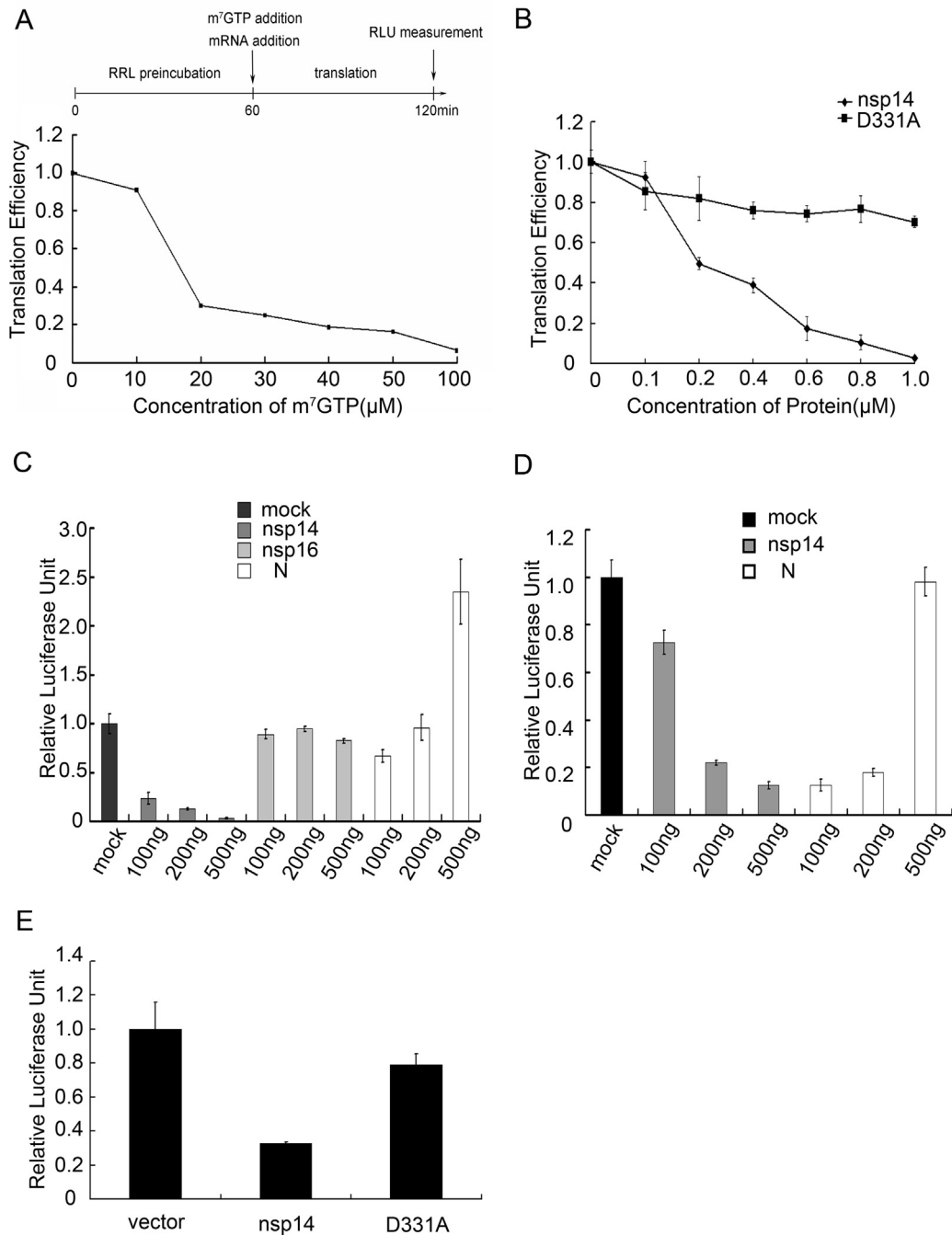


Fig. 6. Inhibition of protein translation by nsp14 of SARS-CoV. (A) RRL was pre-incubated for 60 min and then the luciferase-encoding mRNA and m⁷GTP at the indicated concentrations were added to the reaction mixture, which was then incubated for another 60 min. The luciferase activity was measured. (B) Recombinant protein nsp14 or mutant D331A of SARS-CoV were incubated with RRL for 60 min; then luciferase-encoding mRNA was added to the reaction mixture and incubated for another 60 min. The luciferase activity was measured, and the error bars indicate standard deviation. (C) and (D) The protein expression constructs pRK-nsp14 or pRK-nsp16 and pRK-N at the indicated dose were co-transfected with the Renilla luciferase reporter plasmid pRL-TK (C) or Renilla luciferase reporter plasmid pRL-SV40 (D) into 293T cells grown in 24-well plates. 1 day post-transfection, luciferase activity was measured. (E) Lysates of 293T cells co-transfected with luciferase reporter plasmid and pRK-nsp14 or pRK-D331A were subjected to a luciferase assay. The data are presented with the SD from three independent experiments.

our previous observations on the cap N7-MTase activity of SARS-CoV nsp14. As the N7-methylated GTP is inhibitory to mRNA translation, the present finding provides a clue for further research on the molecular mechanisms of coronavirus replication and pathogenesis.

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