Virus Research 176 (2013) 45–52

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

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

Xu Jin[a], Yu Chen[a], Ying Sun[a], Cong Zeng[a], Yi Wang[a], Jiali Tao[a], Andong Wu[a], Xiao Yu[a], Zhou Zhang[a,1], Jie Tian[a], Deyin Guo[a,b,*]

[a] State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, PR China
[b] Institute of Medical Virology, Wuhan University School of Medicine, Wuhan 430072, PR China

ARTICLE INFO

*Article history:*
Received 30 March 2013
Received in revised form 4 May 2013
Accepted 6 May 2013
Available online 20 May 2013

*Keywords:*
SARS
Coronavirus
nsp14
GTP methylation
Translation

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.


## 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.

* Corresponding author at: The College of Life Sciences, Wuhan University, Wuhan 430072, PR China. Tel.: +86 27 68752506.
*E-mail addresses:* dguo@whu.edu.cn, deyin.guo@gmail.com (D. Guo).
[1] Current address: Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA.

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.05.001

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 subcloned 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 $MgSO_4$, 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 (Genesript) 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 $^{32}$P-labeling were carried out in 10 μl reaction mixture [2 μM of purified recombinant proteins, 0.3 pM of $^{32}$P-labeled GTP, 2 mM of GTP, 40 mM Tris–HCl (pH 7.5 or 8.0), 2 mM $MgCl_2$, 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 $^{32}$P-labeled cap was determined by scanning the chromatogram with a PhosphorImager as described previously (Chen et al., 2009).

The MTase activity assays with $^{3}$H-labeling were carried out in 30 μl reaction mixture [40 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 2 mM DTT, 40 units RNase inhibitor, 0.01 mM AdoMet], with 14.9 pM of Ado[methyl-$^{3}$H]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. $^{3}$H-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 socked 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 $MgCl_2$, 5 mM dithiothreitol, 1.2% n-octyl-β-D-glucopyranoside, 100 μM AdoMet, and 2 μCi of [a-$^{32}$P]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).