Mutagenesis of S-adenosyl-L-methionine-binding Residues in Coronavirus nsp14 N7-Methyltransferase Demonstrates Differing Requirements for Genome Translation and Resistance to Innate Immunity

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Running title: CoV N7-MTase in translation and innate immunity

Keywords: coronavirus, MHV, N7-methyltransferase, N7-MTase, viral capping, RNA capping, innate immunity, translation

Word Count: (248 abstract; 88 importance)

ABSTRACT

Eukaryotic mRNAs possess a methylated 5'-guanosine cap that is required for RNA stability, efficient translation, and protection from cell-intrinsic defenses. Many viruses use 5' caps or other mechanisms to mimic a cap structure to limit detection of viral RNAs by intracellular innate sensors and to direct efficient translation of viral proteins. The coronavirus (CoV) nonstructural protein 14 (nsp14) is a multifunctional protein with N7-methyltransferase (N7-MTase) activity. The highly conserved S-adenosyl-L-methionine (SAM)-binding residues of the DxG motif are required for nsp14 N7-MTase activity *in vitro*. However, the requirement for CoV N7-MTase activity and the importance of the SAM-binding residues during viral replication have not been determined. Here, we engineered mutations in murine hepatitis virus (MHV) nsp14 N7-MTase at residues D330 and G332 and determined the effects of these mutations on viral replication, sensitivity to mutagen, inhibition by type I interferon, and translation efficiency. Virus encoding a G332A substitution in nsp14 displayed delayed replication kinetics and decreased peak titers relative to WT MHV. In addition, replication of nsp14 G332A virus was diminished following treatment of cells with interferon-β, and nsp14 G332A genomes were translated less efficiently both *in vitro* and during viral infection. In contrast, alanine substitution of MHV nsp14 D330 did not affect viral replication, sensitivity to mutagen, or inhibition by interferon-β compared to WT MHV. Our results demonstrate that the conserved MHV N7-MTase SAM-binding site residues are not required for MHV viability and suggest that the determinants of CoV N7-MTase activity differ *in vitro* and during virus infection.

43 **IMPORTANCE**

44 Human coronaviruses, most notably SARS-CoV and MERS-CoV, cause severe and lethal human
45 disease. Since specific antiviral therapies are not available for the treatment of human
46 coronavirus infections, it is essential to understand the functions of conserved CoV proteins in
47 viral replication. Here, we show that alanine substitution of G332 in the N7-MTase domain of
48 nsp14 impairs viral replication, enhances sensitivity to the innate immune response, and reduces
49 viral RNA translation efficiency. Our data support the idea that coronavirus RNA capping could
50 be targeted for development of antiviral therapeutics.

51

52 **INTRODUCTION**

53 Eukaryotic mRNAs possess a methylated 5' guanosine cap linked to the penultimate nucleotide
54 by a 5'-5' triphosphate bridge (1). 5' capping of cellular mRNAs functions in RNA stability, pre-
55 mRNA splicing, mRNA export from the nucleus, translation, and protection against cellular
56 antiviral defenses (2). The canonical cellular capping process involves three enzymes: 1) an
57 RNA triphosphatase (RTPase), which is responsible for cleaving the γ-phosphate of the nascent
58 transcript, 2) a guanylyltransferase (GTase), which transfers a guanosine monophosphate (GMP)
59 moiety to the 5' diphosphate RNA, and 3) an N7-methyltransferase (N7-MTase), which is
60 responsible for transferring a methyl group from the methyl donor, S-adenosyl-L-methionine
61 (SAM), to the N7 position of the guanosine base (3). These sequential reactions lead to
62 formation of a cap-0 (7-methyl-Gppp) structure, which is thought to be the minimal cap
63 determinant required for eIF4E recognition and efficient translation (4-6). Higher eukaryotes
64 express 2'O-methyltransferases (2'O-MTase) that add a methyl group to the ribose 2'O position
65 of the penultimate nucleotide of the cap-0 RNA. This reaction results in formation of a cap-1
66 structure that allows cells to differentiate self from non-self RNAs in the cytoplasm (7, 8).
67 Eukaryotic viruses use host translation machinery, and many of these viruses encode
68 capping enzymes. The diversity of enzymes and mechanisms used by viruses to synthesize
69 capped RNA products suggests that there is selective pressure on viruses to cap their RNAs (9).
70 Coronaviruses (CoVs) encode several enzymes within their large, positive-sense RNA genomes
71 (27-34 kb) that are implicated in viral RNA capping. The coronavirus genome possesses a 5'
72 terminal cap and 3' poly-A tail (10-12). All data to date support the hypothesis that CoV
73 genomes are capped using the canonical mRNA capping pathway (13). Severe acute respiratory
74 syndrome coronavirus (SARS-CoV) nsp13 displays RTPase activity *in vitro* (14). The CoV

guanylyltransferase has not been identified but, according to the current model, would function to add a GMP to the diphosphate RNA product of nsp13. The RNA-dependent RNA polymerase (RdRp) of equine arteritis virus and SARS-CoV displays nucleotidylation activity (15). While further study is required to define the function of this activity in viral replication, it is possible that the RdRp participates in CoV RNA capping. Nsp16 of feline coronavirus functions independently as a 2'O-MTase (16-18), but SARS-CoV nsp16 requires nsp10 as a co-factor for 2'O-MTase activity. SARS-CoVs lacking 2'O-MTase activity are recognized and sequestered by IFIT1 (13, 19-23) due to the lack of a cap-1 structure.

CoV nsp14 is a multifunctional protein with 3'-5' exoribonuclease activity and N7-MTase activity (24, 25). Nsp14-mediated N7-methylation of Gppp-RNA to form a cap-0 structure is a prerequisite for nsp10/16-mediated 2'O-methylation *in vitro* (13). A conserved DxG motif within the MTase domain is required for SAM-binding *in vitro*, and alteration of these residues abolishes MTase activity *in vitro* (13, 26). However, the requirements of the CoV nsp14 N7-MTase during viral replication are not known. Therefore, we assessed the effect of mutations in the DxG motif of the MHV nsp14 N7-MTase on viral replication. We show that alanine substitution of nsp14 D330 does not alter viral replication kinetics or increase sensitivity to interferon-β treatment relative to wild-type (WT) MHV. However, alanine substitution of nsp14 G332 impaired virus replication, resulting in delayed replication kinetics and decreased peak titer, relative to WT MHV. In addition, nsp14 G332A virus displayed increased sensitivity to treatment of cells with interferon-β, and nsp14 G332A genomes were translated less efficiently *in vitro* and during infection. These data suggest that residue G332, but not residue D330, is required for MHV nsp14 N7-MTase activity, and collectively, that the regulation of

97 CoV capping is likely more complex in the context of replicating virus than during *in vitro*

98 biochemical studies with isolated proteins.

99

100 **MATERIALS AND METHODS**

101 **Cells and viruses.** Murine delayed brain tumor (DBT) cells (27) and baby hamster kidney 21
102 cells expressing the MHV receptor (BHK-R) (28) were maintained at 37°C in Dulbecco's
103 modified Eagle medium (DMEM; Gibco) supplemented to contain 10% fetal bovine serum
104 (FBS;Invitrogen), 100 U/ml penicillin and streptomycin (Gibco), and 0.25 µg/ml amphotericin B
105 (Corning). BHK-R cells were further supplemented to contain 0.8 mg/ml of G418 (Mediatech).
106 Bone-marrow-derived dendritic cells (BMDCs) were maintained in R10 medium (RPMI 1640
107 [Gibco] supplemented to contain 10% FBS, 2 mM L-glutamine, 100 µg/ml gentamicin [MP
108 Biomedicals], 0.25 µg/ml amphotericin B, 50 µM beta-mercaptoethanol, 20 ng/ml GM-CSF, and
109 10 ng/ml IL-4). Recombinant MHV strain A59 (GenBank accession number AY910861) was
110 propagated as described (28).
111
112 **Cloning, recovery, and verification of mutant viruses.** Site-directed mutagenesis was used to
113 engineer point mutations in individual MHV genome cDNA fragment plasmids using the MHV
114 infectious clone reverse genetics system (28). Viruses encoding firefly luciferase (FFL) fused to
115 nsp2 were recovered using MHV A frag-FFL2 (29). Mutant viruses were recovered using BHK-
116 R cells following electroporation of *in vitro*-transcribed genomic RNA. All mutagenized
117 plasmids were sequenced (GenHunter Corporation, Nashville, TN) to ensure that no additional
118 mutations were introduced. Recovered viruses also were sequenced to verify the engineered
119 mutations.
120
121 **Virus replication kinetics.** Sub-confluent DBT cell monolayers were infected at a multiplicity
122 of infection (MOI) of 1 plaque-forming unit (PFU) per cell at 37°C for 45 min. Inocula were

123 removed, cells were washed with 1X PBS, and fresh medium was added. Aliquots were
124 harvested at various times post-infection. Viral titer at various intervals was determined by
125 plaque assay (30).
126
127 **5-FU sensitivity assays.** 5-fluorouracil (5-FU, Sigma) was prepared as a 200 mM stock solution
128 in DMSO. Sub-confluent DBT cells were treated with DMEM supplemented to contain various
129 concentrations of 5-FU or DMSO alone at 37°C for 30 min (31). Drug was removed, and cells
130 were infected with virus at an MOI of 0.01 PFU/cell at 37°C for 1 h. Inocula were removed, and
131 cells were incubated in medium containing 5-FU or DMSO. Cell culture supernatants were
132 collected at 24 h post-infection, and viral titers were determined by plaque assay.
133
134 **Interferon-β sensitivity assays.** Sub-confluent DBT cells were treated with various
135 concentrations of mouse interferon-β (IFN-β, PBL Assay Science) for 18 h prior to infection
136 with virus at an MOI of 1 PFU/cell at 37°C for 45 min. Inocula were removed, cells were
137 washed with PBS, and fresh medium was added. Cell culture supernatants were collected at
138 indicated times post-infection, and viral titers were determined by plaque assay.
139
140 **Interferon-β induction assays.** Sub-confluent DBT cells were treated with 10 U/ml mouse IFN-
141 β for 18 h prior to infection with virus at an MOI of 0.1 PFU/cell at 37°C for 45 min. Inocula
142 were removed, cells were washed with PBS, and fresh medium was added. At 12 h post-
143 infection, cell culture supernatants were aspirated and cell lysates were harvested by adding
144 TRIzol reagent. Total RNA present in lysates was purified using the phenol/chloroform method.
145 cDNA was generated by RT-PCR using 1μg of total RNA as described (31). Mouse IFN-β

146 expression levels relative to GAPDH were determined by qPCR using the Applied Biosciences
147 7500 Real-Time PCR System with Power SYBR Green PCR Master Mix and IFN-β primers:
148 FWD: 5'-TCCGCCCTGTAGGTGAGGTTGAT-3' and REV: 5'-
149 GTTCCTGCTGTGCTTCTCCACCA-3' and GAPDH primers previously reported (31).
150
151 **Generation and infection of BMDCs**. Primary BMDCs were isolated from the hind limbs of
152 WT and IFN-α/β receptor-deficient (IFNAR$^{-/-}$) C57BL/6J mice. Mice were euthanized by
153 isoflurane overdose, and hind limbs were resected. Bone marrow cells were collected by flushing
154 the femurs and tibiae with medium. Cells were strained through a 70-μm cell strainer, and red
155 blood cells were lysed. Cells were cultured at 37°C in R10 medium supplemented to contain 20
156 ng/ml GM-CSF and 10 ng/ml IL-4. At 3 d post-plating, cell culture supernatants were removed
157 and replaced with fresh R10 medium. Six days post-plating, cells were lifted using Cellstripper
158 (Corning) and replated with fresh R10 medium in 24-well plates at a density of 10^5 cells/well and
159 incubated at 37°C overnight. WT and IFNAR$^{-/-}$ BMDCs were infected with virus at an MOI of
160 0.01 PFU/cell at 37°C for 45 min. Inocula were removed, and fresh medium was added. Cell
161 culture supernatants were collected 24 h post-infection, and viral titers were determined by
162 plaque assay. All experiments with animals were performed in accordance with Vanderbilt
163 University School of Medicine Institutional Animal Care and Use Committee guidelines.
164
165 **Purification of virions and extraction of RNA.** Virion RNA was purified from sub-confluent
166 T150 flasks of BHK-R cells infected with WT-FFL or nsp14 G332A-FFL viruses at an MOI of
167 0.001 PFU/cell. When CPE was apparent throughout the monolayer, cell culture supernatants
168 were collected and pooled into 50 ml conical tubes (Corning), clarified by centrifugation at 1,000

x *g* for 10 min, and stored at -80°C. Upon thawing, virus particles in the clarified supernatants were collected by ultra-centrifugation at 106,750 x *g* overnight through a 5 ml, 20% (w/w) sucrose cushion in an SW32Ti rotor. The pelleted particles were resuspended in 200 µl MSE buffer (10 mM MOPS [pH 6.8], 150 mM NaCl, 1 mM EDTA) and incubated at 4°C overnight prior to resuspension by gently pipetting several times. Viral RNA was isolated from purified viral particles using TRIzol reagent (Invitrogen) and phenol/chloroform extraction.

***In vitro* translation reactions.** Viral genomic RNAs containing an in-frame firefly luciferase encoding sequence were translated at 30°C for various intervals in 10 µl of rabbit reticulocyte lysate (Promega) in the presence of both 10 µM amino acid mixture minus leucine and 10 µM amino acid mixture minus methionine.

Firefly luciferase assays. Sub-confluent DBT cells were infected with virus at an MOI of 0.1 PFU/cell. At various intervals, cell culture supernatants were removed, cells were washed with PBS, and 100 µl of reporter cell lysis buffer (Promega) was added to each well. Cells lysates were frozen at -80°C to promote lysis and thawed at room temperature prior to quantifying firefly luciferase activity. Luciferase activity from cell lysates or *in vitro* translation reactions was quantified using a Veritas luminometer (Turner Biosystems) and the firefly luciferase assay system (Promega).

Determination of specific infectivity. Sub-confluent monolayers of DBT-9 cells were infected with virus at an MOI of 0.1 PFU/cell at 37°C for 45 min. Inocula were removed, fresh medium was added, and cells were incubated at 37°C for 24 h. Cell culture supernatants were collected,

192 and viral titers were determined by plaque assay. Supernatants also were used for RNA genome
193 isolation by adding 100 µl supernatant to 900 µl TRIzol reagent, chloroform extraction by phase
194 separation, and final purification using the PureLink Mini RNA kit (Ambion). Genome RNA
195 was quantified using one-step qRT-PCR, and the particle to PFU ratio was calculated.
196
197 **Genome RNA stability assay.** Sub –confluent monolayers of DBT-9 cells were infected with
198 virus at an MOI of 0.01 PFU/cell at 37°C for 45 min in the presence of DMSO or 100 µg/ml
199 cycloheximide (CHX) (Sigma). Inocula were removed, media containing DMSO or 100 µg/ml
200 CHX was added, and cell lysates were harvested at indicated times post-infection by removing
201 the cell culture supernatant and adding TRIzol reagent. Lysates were spiked with a known
202 amount of *in vitro* transcribed *Renilla* luciferase RNA and total RNA was obtained by phenol/
203 chloroform extraction. cDNA was generated by RT-PCR and viral genome copies present
204 relative to *Renilla* luciferase was determined by SYBR Green qPCR using nsp10 (31) and
205 *Renilla* luciferase specific primers (22)**.**
206
207 **Quantification of viral genomic RNA by qRT-PCR.** An RNA standard was prepared using the
208 MHV A fragment (28) to generate a 931 nucleotide RNA. First, cDNA was generated by PCR
209 amplification using the primers: forward 5'-
210 TAATACGACTCACTATAGGGGGCTATGTGGATTGTTGTGG-3', which initiates with a T7
211 promoter, and reverse 5'-AATTCTTGACAAGCTCAGGC-3'. RNA for the standard curve was
212 prepared using an mMessage mMachine T7 kit (Ambion) and purified using an RNeasy Mini kit
213 (Qiagen). A standard curve was generated using 10-fold dilutions from 10^3 to 10^8 copies. A 5' 6-
214 carboxyfluorescein (FAM)-labeled probe (5'-TTCTGACAACGGCTACACCCAACG-3'

[Biosearch Technologies]) was used with forward (5'-AGAAGGTTACTGGCAACTG-3') and reverse (5'-TGTCCACGGCTAAATCAAAC-3') primers. Reactions were incubated on ice with enzyme added last. Final volume for reactions was 20 µl with 150 nM probe, 900 nM each primer, 2 µl sample RNA, and 10 µl 2X ToughMix, one-step, low ROX enzyme mix (Quantas) per reaction. Samples were quantified in duplicate using an Applied Biosciences 7500 Real-Time PCR System with the conditions 55°C for 10 min, 95°C for 5 min, 95°C for 30 s, and 60°C for 1 min, with the last two steps repeated 40 times. The standard curve was plotted using GraphPad Prism 6 software, and genomes/µl were calculated.

Statistical analysis. Statistical tests were conduced using GraphPad Prism 6 software (La Jolla, CA) as indicated in the respective figure legends.

227 **RESULTS**

228 **Recovery and replication kinetics of MHV nsp14 N7-MTase mutants.** The DxG SAM-
229 binding motif is conserved among the nsp14 N7-MTase domains of alpha-, beta-, and
230 gammacoronaviruses (Fig. 1A). Mutations in this motif of SARS-CoV nsp14 ablate N7-MTase
231 activity of purified proteins *in vitro* (13, 25, 26). To determine whether this motif is required for
232 viral replication, we engineered alanine substitutions at the DxG SAM-binding motif in the
233 MHV nsp14 N7-MTase domain. Virus containing either a D330A or G332A substitution in
234 nsp14 was recovered and sequence confirmed across the nsp14 coding region. Following
235 infection of DBT cells at an MOI of 1 PFU/cell, nsp14 D330A virus replicated with kinetics
236 comparable to WT MHV (Fig. 1B). Nsp14 D330A plaque morphology also was similar to that of
237 WT MHV (Fig. 1C). In contrast, the nsp14 G332A virus began exponential replication 4-6 h
238 later than WT MHV and reached a lower peak titer (1.5×10^4 PFU/ml) relative to WT MHV (10^7
239 PFU/ml) (Fig. 1B). The nsp14 G332A virus plaque size was also decreased relative to WT MHV
240 (Fig. 1C). Thus, despite the requirement of D330 for nsp14 N7-MTase activity *in vitro* (13, 25,
241 26, 32), our data indicate that the D330A mutation has no detectable effect on MHV replication
242 kinetics in cell culture.
243
244 **Nsp14 D330A or G332A mutations do not significantly influence nsp14 ExoN activity.**
245 Coronavirus nsp14 is a multifunctional protein with two known enzymatic activities, a
246 proofreading 3'-5' exoribonuclease activity (ExoN) and N7-MTase activity (24, 25). Based on *in*
247 *vitro* studies, the ExoN and N7-MTase domains of CoV nsp14 are interdependent (26). This
248 conclusion is supported by the crystal structure of nsp14, demonstrating that the ExoN and N7-
249 MTase domains interact through a large hydrophobic interface (32). In addition, disruption of

ExoN (ExoN-) via mutations at two active-site residues decreases replication fidelity of MHV and SARS-CoV and renders the viruses sensitive to the RNA mutagen 5-fluorouracil (5-FU) (30, 31, 33). Thus, 5-FU sensitivity has been shown to be an *in vitro* indicator of ExoN activity. Therefore, we tested whether the D330A or G332A mutations affect ExoN activity by treating cells with increasing concentrations of 5-FU or vehicle (DMSO) prior to infection with either nsp14 D330A or nsp14 G332A virus at an MOI of 0.01 PFU/cell (Fig. 2). The nsp14 D330A and nsp14 G332A viruses were not significantly altered in 5-FU sensitivity compared with WT MHV (N.S. by One-way ANOVA). In contrast, the ExoN- virus displayed a concentration-dependent increase in 5-FU sensitivity. These results indicate that neither D330A nor G332A significantly alter ExoN activity during virus replication.

MHV nsp14 G332A is detected by and sensitive to the type I interferon-mediated innate immune response. Coronavirus RNA capping likely follows the conventional capping pathway, with nsp14 N7-methylation being a prerequisite for 2'O-methylation *in vitro* (13). Therefore, decreased nsp14 N7-MTase activity should reduce overall 2'O-methylation, thereby increasing virus sensitivity to exogenous type I IFN due to recognition by IFIT1 and MDA5 (22, 23). To test this hypothesis, we pretreated DBT cells with murine IFN-β prior to infection with WT MHV, nsp16 D130A, an IFN-sensitive positive control due to ablated 2'O-MTase activity (19, 22, 23, 34), or nsp14 D330A or nsp14 G332A N7-MTase mutant viruses at an MOI of 1 PFU/cell. Cell culture supernatants were collected at either 12 or 24 h post-infection, and viral titers were determined by plaque assay. As expected, the nsp16 D130A virus was sensitive to IFN-β pretreatment (Fig. 3A). The nsp14 G332A virus demonstrated a dose-dependent increase in IFN-β sensitivity, which became undetectable by plaque assay at IFN-β concentrations greater

than 75 U/ml (Fig. 3A). In contrast, nsp14 D330A virus displayed sensitivity to IFN-β comparable to WT MHV (Fig. 3B). Because nsp14 D330A displayed replication kinetics and resistance to IFN-β pre-treatment indistinguishable from WT MHV, it is likely that the D330A substitution does not significantly affect N7-MTase activity. Therefore, we focused solely on the nsp14 G332A mutant for the remainder of the experiments in this study.

In addition to an increased sensitivity to the effects of type I interferon pretreatment, coronaviruses lacking 2'O-MTase activity induce higher levels of IFN-β than WT (8, 22, 23). Therefore, to determine whether nsp14 G332A is also recognized by innate sensors and subsequently induces type I interferon expression, we pretreated DBT cells with 10 U/mL murine IFN-β for 18 h prior to infection with WT MHV, nsp16 D130A, nsp14 G332A viruses at an MOI of 0.1 PFU/cell. At 12 h post-infection, cell lysates were collected and the relative expression of IFN-β determined by qPCR (Fig. 3C). As previously reported, infection with WT MHV marginally induced the expression of IFN-β (35) and infection with nsp16 D130A led to an up-regulation of IFN-β relative to mock infected cells (8, 22, 23). Furthermore, infection with nsp14 G332A led to a significant increase in the expression of IFN-β relative to mock and WT MHV infected cells. These data further suggest that nsp14 N7-MTase activity precedes nsp16 2'O-MTase activity and the absence of either activity results in innate detection of the virus leading to the induction of type I interferon gene expression.

To determine the effect that increased sensitivity to IFN-β has on nsp14 G332A replication, we tested whether nsp14 G332A virus replication could be rescued in BMDCs lacking the IFN alpha/beta receptor ($IFNAR^{-/-}$). $IFNAR^{-/-}$ cells lack the capacity to respond to type I IFNs and, thus, are incapable of mounting an effective IFN-dependent antiviral response (36). WT or $IFNAR^{-/-}$ BMDCs were infected with WT MHV or nsp14 G332A virus at an MOI of

296 0.01 PFU/cell, cell culture supernatants were collected 24 h post-infection, and viral titers were
297 determined by plaque assay. Similar to experiments using DBT cells, nsp14 G332A virus
298 replicated poorly in WT BMDCs relative to WT MHV (Fig. 3D). Titers of nsp14 G332A virus
299 were increased by approximately 40-fold in IFNAR$^{-/-}$ BMDCs (5.6 x 10^4 PFU/ml) compared
300 with the titers of this virus in WT BMDCs (1.3 x 10^3 PFU/ml). However, despite the increase in
301 viral titers of nsp14 G332A in IFNAR$^{-/-}$ BMDCs, titers were not restored to the level of WT
302 MHV in IFNAR$^{-/-}$ BMDCs (3.6 x 10^6 PFU/ml). These data suggest that the impaired replication
303 capacity of nsp14 G332A virus is only in part attributable to IFN sensitivity and, instead, this
304 virus may manifest a more general replication defect.
305
306 **Nsp14 G332A genome translation is delayed during infection.** Since the absence of the
307 IFNAR was insufficient to restore nsp14 G332A replication, other mechanisms, such as
308 decreased genome RNA stability or decreased viral genome translation, may contribute to the
309 replication defect of this virus. 5' capping of cellular mRNAs serves several important functions,
310 one of which is to increase RNA stability (2, 9). To test the stability of the nsp14 G332A genome
311 upon entry into the cell, we infected DBT cells with WT MHV or nsp14 G332A virus at an MOI
312 of 0.01 PFU/cell in the presence of vehicle (DMSO) or 100 µg/ml cycloheximide (CHX). CHX
313 inhibits the translation of input viral genomes and prevents expression of the viral RNA
314 dependent RNA polymerase, thereby allowing us to quantify the amount of coronavirus RNA
315 present at later time-points relative to input. At the indicated times post-infection, cell lysates
316 were collected, spiked with a known amount of *in vitro* transcribed *Renilla* luciferase, and the
317 amount of viral RNA present relative to *Renilla* luciferase determined by qPCR (Fig. 4). At each
318 time-point post-infection for CHX treated samples, the level of nsp14 G332A RNA was similar

to WT MHV, indicating that nsp14 G332A replication is not impaired due to decreased genome RNA stability.

In addition to serving as a precursor for 2'O-methylation, N7-methylated guanosine 5' caps are recognized by eIF4E and required for efficient translation of eukaryotic RNA (9, 37). To determine whether the nsp14 G332A mutation impairs viral translation efficiency, we first engineered virus encoding FFL as an in-frame N-terminal fusion with MHV nsp2 (29) in the ORF1a polyprotein coding sequence of the isogenic nsp14 G332A cloned genome. In this setting, FFL-nsp2 is the second protein translated from the input viral genome and becomes a reporter for viral protein translation. We infected DBT cells with either WT-FFL or nsp14 G332A-FFL virus at an MOI of 0.1 PFU/cell, and lysates were prepared at various intervals post-infection to quantify luciferase activity and viral genome RNA copy number. Luciferase activity accumulated more slowly following infection by nsp14 G332A-FFL virus relative to WT-FFL virus (Fig. 5A). WT-FFL signal began to decline after 16 h due to destruction of the cell monolayer. In addition, levels of nsp14 G332A-FFL genomic RNA increased more slowly than those of WT-FFL (Fig. 5B). By quantifying both luciferase activity and viral genome copies, we were able to calculate the kinetics of translation. To determine the rate of translation at each time-point post-infection, the ratio of luciferase activity to genome copies was determined using data from Figs. 5A and 5B. The ratio of luciferase activity to genome copies for WT-FFL was highest at early times post-infection (Fig. 5C). In contrast, the ratio of luciferase activity to genome copies was substantially less for the nsp14 G332A-FFL virus at early time-points post-infection compared to WT-FFL and failed to reach peak WT-FFL levels. These data demonstrate that nsp14 G332A-FFL virus requires more genomic RNA to achieve WT levels of FFL activity, consistent with decreased translation efficiency of the mutant virus genome. Therefore, we next

342 determined whether nsp14 G332A-FFL and WT-FFL virions are equivalently infectious by
343 measuring the specific infectivity of each virus from infected DBT cell culture supernatants. The
344 ratio of nsp14 G332A-FFL particles per PFU was approximately 7-fold more than WT-FFL (Fig.
345 5D). Thus, packaged nsp14 G332A-FFL genomes were less efficient at establishing infection
346 than WT.
347
348 **Nsp14 G332A-FFL genomes are translated less efficiently than WT-FFL genomes *in vitro*.**
349 To directly assess the translation capacity of nsp14 G332A-FFL virus genomes, we isolated
350 genome RNA from purified virions. Increasing concentrations of genome RNAs were incubated
351 with rabbit reticulocyte lysates at 30°C for 1.5 h, and luciferase activity was quantified (Fig. 6A).
352 Compared to WT-FFL genomes, FFL activity in the reticulocyte lysates was significantly
353 reduced following incubation with nsp14 G332A-FFL genomes. In addition, we quantified the
354 relative translation efficiency of equal amounts of WT-FFL and G332A-FFL genomic RNA over
355 time. At all time points tested after 15 min, FFL activity was significantly reduced following
356 incubation of reticulocyte lysates with nsp14 G332A-FFL genomes relative to WT-FFL genomes
357 (Fig. 6B). Taken together, our data indicate that the decreased replication capacity of the nsp14
358 G332A virus is attributable to IFN sensitivity and reduced translation efficiency.
359

360 **DISCUSSION**

361 In this study, we engineered recombinant CoVs encoding alanine substitutions in the nsp14 N7-
362 MTase at the SAM-binding site residues, D330 and G332. We found that the N7-MTase SAM-
363 binding site mutants are viable and yield drastically different phenotypes during replication.
364 Specifically, MHV nsp14 D330A virus replicates indistinguishably from WT MHV in all assays
365 conducted, despite the requirement of this residue for SAM binding *in vitro* (26). There is
366 precedent for such a contradiction. A previous study using vesicular stomatitis virus identified a
367 SAM-binding residue within the L protein (G1674) that, when altered, does not affect viral
368 replication or N7-MTase activity (38). The structure of the SARS-CoV nsp10-nsp14 complex
369 reveals that D331 (D330 in MHV) is in close proximity to the SAM-binding site, but only G333
370 (G332 in MHV) directly contacts SAM (32). Since *in vitro* N7-MTase activity was assessed only
371 for a SARS-CoV nsp14 D331A/G333A double mutant, it is not clear whether nsp14 D331 was
372 required for N7-MTase activity in this study (32). However, a previous study using both *in vitro*
373 functional assays and yeast complementation reported that SARS-CoV nsp14 D331 is essential
374 for N7-MTase activity (26). Our study examined nsp14 N7-MTase in the context of viral
375 replication. A potential difference between our work and previous studies of the CoV nsp14 N7-
376 MTase is the use of MHV versus SARS-CoV proteins, respectively. Purified MHV nsp14 N7-
377 MTase is not available in our lab for biochemical studies. However, our results will guide future
378 experiments when such a system is established. During our study, we attempted to recover
379 SARS-CoV nsp14 D331A, I332A, and G333A N7-MTase mutant viruses. However, viable
380 viruses were not recovered after at least three attempts for each mutant. Nonetheless, the high
381 conservation of the SAM-binding residues makes it unlikely that the differences observed
382 between our work and previous biochemical studies are due to profoundly different N7-MTase

383 catalytic mechanisms.

384 In contrast to nsp14 D330A virus, nsp14 G332A virus replicated with delayed kinetics
385 and reached peak titers that were 1000-fold less than those of WT MHV. CoV nsp14 has two
386 domains: an N-terminal ExoN domain and a C-terminal N7-MTase domain. Mutations at D331
387 in SARS-CoV nsp14 do not affect ExoN activity *in vitro* (25, 26). However, the effect of altering
388 residue G333 (G332 in MHV) on ExoN activity has not been reported using any system. It is
389 unlikely that the G332A mutation in MHV nsp14 influences ExoN activity, as nsp14 G332A
390 demonstrated WT-like sensitivity to the RNA mutagen, 5-FU. Even a subtle alteration in ExoN
391 activity should result in a detectable change in 5-FU sensitivity, particularly since we performed
392 the assay using low-MOI conditions, which would increase mutagen incorporation during multi-
393 step replication (31, 34). The lack of enhanced 5-FU sensitivity for the nsp14 D330A and nsp14
394 G332A viruses indicates that mutations at these SAM-binding residues do not significantly
395 dampen ExoN activity during virus replication. Additionally, since nsp14 G332A is resistant to
396 5-FU treatment, it is unlikely that the G332A phenotype is due to nsp14 instability or
397 degradation.

398 Our data indicate that impaired replication of nsp14 G332A virus is likely due to a
399 combination of factors, including increased detection by innate immune sensors and decreased
400 translation efficiency of viral RNA. Binding of type I IFNs to the IFN receptor leads to
401 expression of many IFN-stimulated genes and ultimately the establishment of an antiviral state
402 (39). Coronavirus RNAs lacking 2'O-methylation are sensed by IFIT1, which is one of the most
403 highly up-regulated IFN-stimulated genes following IFN induction (40). While nsp14 D330A
404 displayed WT-like sensitivity to pretreatment with IFN-β, nsp14 G332A virus did not replicate
405 following IFN-β pretreatment with doses greater than 75 U/ml. However, initial titers were lower

406 for nsp14 G332A. Thus, the concentration-dependent change in viral titer following IFN-β
407 pretreatment was similar to the nsp16 D130A virus. The IFN-β sensitivity of nsp14 G332A
408 likely results from a reduction in 2'O-methylation of viral RNA due to impaired N7-MTase
409 activity. This hypothesis is supported by our data showing that infection with either nsp16
410 D130A or nsp14 G332A virus results in the induction of IFN-β gene expression. In addition,
411 decreased N7-MTase activity due to the G332A mutation results in the delayed translation and
412 decreased translation efficiency observed during viral replication and *in vitro* assays. Due to the
413 highly impaired replication capacity of the nsp14 G332A virus, it has not been possible to
414 directly determine the cap methylation status of nsp14 G332A virus genomes. Nevertheless, our
415 results are consistent with functions of the N7-methylated 5'cap in promoting both viral and
416 cellular translation (4-6). Decreased translation efficiency also could explain the lower specific
417 infectivity observed for nsp14 G332A virus. Furthermore, it is possible that the delayed
418 translation kinetics of nsp14 G332A genomic RNA increases innate sensing of the virus by
419 delaying the early expression of multiple CoV IFN antagonists upon entry, resulting in decreased
420 replication capacity.

421 Our data provide additional support for a sequential model of CoV RNA capping
422 wherein N7-methylation precedes 2'O-methylation. In addition, our studies suggest that small-
423 molecule inhibitors of the CoV nsp14 N7-MTase would impair virus replication and provide a
424 pathogen-associated molecular pattern that would be quickly recognized by the innate immune
425 response. Given the conservation of these enzymes, such inhibitors may have activity against
426 diverse groups of coronaviruses.
427

428 **ACKNOWLEDGMENTS**

429 We thank Clint Smith for critical review of the manuscript and members of the
430 Denison and Dermody laboratories for useful discussions. This work was supported by Public
431 Health Service awards T32 HL07751 (J.B.C. and A.W.A.) from the National Heart, Lung, and
432 Blood Institute and R01 AI038296 (T.S.D.) and R01 AI108197 (M.R.D.) from the National
433 Institute of Allergy and Infectious Diseases. Additional support was provided by the Elizabeth B.
434 Lamb Center for Pediatric Research.

435

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FIGURE LEGENDS

FIG 1 Replication kinetics of viruses with altered N7-MTase SAM-binding residues. (A) Alignment of GenBank ORF1b sequences of the α-, β-, and γ-CoVs shown demonstrates that SAM-binding residues (shaded) are highly conserved. (B) DBT cells were infected with the viruses shown at an MOI of 1 PFU/cell. Cell culture supernatants were collected at the indicated times post-infection, and viral titers were determined by plaque assay. Error bars indicate SEM (n = 6). (C) Plaque morphology of the viruses shown following agarose overlay plaque assay and fixation with 3.7% paraformaldehyde 24 h post-infection.

FIG 2 N7-MTase mutants display WT-like sensitivity to the RNA mutagen, 5-FU. DBT cells

were treated with the indicated concentrations of 5-FU for 30 min prior to infection with the viruses shown at an MOI of 0.01 PFU/cell. Medium containing 5-FU or vehicle was added 30 min post-infection. After 24 h, cell culture supernatants were collected, and viral titers were determined by plaque assay. For each virus, titers were normalized to those following infection of DMSO-treated controls. Change in viral titer for nsp14 D330A and nsp14 G332A viruses were not statistically significant relative to WT MHV by one-way ANOVA. Error bars indicate SEM (n = 4).

FIG 3 Nsp14 G332A virus exhibits increased induction of and sensitivity to IFN-β. DBT cells were treated for 18 h with the indicated concentrations of mouse IFN-β. Cells were infected with WT, nsp16 D130A, or nsp14 G332A virus and incubated for 24 h (A) or infected with WT, nsp16 D130A, and nsp14 D330A virus and incubated for 12 h (B). Cell culture supernatants were collected, and viral titers were determined by plaque assay. For each panel, error bars represent SEM (n = 4). ND = not detectable. C) DBT cells were treated for 18 h with 10 U/ml mouse IFN-β. Cells were mock infected or infected with WT, nsp16 D130A, or nsp14 G332A virus at an MOI of 0.1 PFU/cell. At 12 h post-infection, cell lysates were harvested, total RNA extracted, cDNA generated, and IFN-β expression relative to GAPDH determined by qPCR. Error bars indicate SEM (n=9). N.S. = not significant, **, P <0.01 by Student's t-test. (D) BMDCs were infected with either WT or nsp14 G332A virus at an MOI of 0.01 PFU/cell. At 24 h post-infection, cell culture supernatants were collected, and viral titers were determined by plaque assay. Error bars indicate SEM (n = 6). *, $P < 0.05$, ***, $P < 0.001$ by Student's t-test.

FIG 4 Nsp14 G332A genomic RNAs are stable. DBT cells were infected with WT or nsp14

G332A virus at an MOI of 0.01 PFU/cell in the presence of vehicle (DMSO) or 100 µg/ml CHX. Cell lysates were harvested at indicated times post-infection, spiked with a known amount of *in vitro* transcribed *Renilla* luciferase RNA, and total RNA obtained by phenol/ chloroform extraction. cDNA was generated by RT-PCR and viral genome copies present relative to *Renilla* luciferase was determined by SYBR Green qPCR using MHV nsp10 and *Renilla* luciferase specific primers. Error bars indicate SEM (n=6).

FIG 5 Nsp14 G332A genomic RNAs are translated with delayed kinetics during infection. DBT cells were infected with either WT-FFL or nsp14 G332A-FFL virus at an MOI of 0.1 PFU/cell. At the times shown post-infection, cell culture supernatants were collected, and lysates were harvested and divided equally into two samples. For the first lysate sample, luciferase activity was quantified (A). For the remaining lysate sample, RNA was extracted, and genome RNA copies were quantified using real-time qRT-PCR with a standard curve and CoV nsp2-specific primers (B). (C) Translation of WT-FFL or nsp14 G332A-FFL genomes at the times shown post-infection as determined by luciferase activity per genome RNA copy number. Values were normalized to WT-FFL at 6 h post-infection. Error bars indicate SEM (n = 4). (D) Viral titers in cell culture supernatants from DBT cells infected with either WT-FFL or nsp14 G332A-FFL were determined by plaque assay and the number of genome RNA copies present in the input supernatant was determined by one-step real-time qRT-PCR. The particle to PFU ratio was calculated by dividing the number of genome RNA copies by viral titers. Error bars represent SEM (n = 4). **, $P < 0.01$ by Student's *t*-test.

FIG 6 Purified nsp14 G332A genomic RNA is translated at lower efficiency *in vitro*. BHK-R

610 cells were infected at an MOI of 0.001 PFU/cell with either WT-FFL or nsp14 G332A-FFL
611 virus. Supernatants were harvested and clarified, and virions were collected by
612 ultracentrifugation. Virion pellets were resuspended, TRIzol was added, and virion RNAs were
613 purified using phenol/chloroform phase separation. Genome RNA copies were quantified using
614 one-step real-time qRT-PCR with a standard curve and CoV nsp2-specific primers. (A) The
615 concentrations of WT-FFL or G332A-FFL genomic RNAs shown were translated *in vitro* at
616 30°C for 1.5 h, and luciferase activity was quantified. Translation values are relative to WT-FFL
617 genomic RNA at 40 ng. Error bars represent SEM (n = 4). ***, $P < 0.001$ by Student's *t*-test. (B)
618 Equivalent numbers of either WT-FFL or nsp14 G332A-FFL genomic RNAs were translated *in*
619 *vitro* for the times shown, and luciferase activity was quantified. Error bars represent SEM (n =
620 6). *, $P < 0.05$, **, $P < 0.01$ by Student's *t*-test.