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CORONAVIRAL 2'-O-METHYLTRANSFERASE FUNCTION IN VIRUS REPLICATION.

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ABSTRACT

Primary and higher order RNA structures in the 5' and 3' untranslated regions of the genomes of positive-strand RNA viruses are known to function as *cis*-acting elements for genome translation and replication. Four *cis*-acting replication elements have been identified in the bovine coronavirus (BCoV) 3' untranslated region that are postulated to recruit viral and cellular proteins for assembly of the replication complex and initiation of negative-strand synthesis. This study had the goal of identifying and establishing the function of the viral proteins that bind BCoV 3' terminal *cis*-acting RNA structures. A major surprising discovery was a specific interaction of the BCoV-encoded 2'-O-methyltransferase (2'-O-MT), an enzyme normally involved in 5'-terminal cap methylation, with a 3'-terminal *cis*-acting octamer-associated bulged stem-loop. This discovery led to detailed analyses of BCoV 2'-O-MT that is summarized as follows: (i) Enzyme probing revealed the higher-order structure of the *cis*-acting 3'-terminal bulged stem loop. (ii) Specific binding of the 2'-O-MT to both the 5' cap-0 structure and to the 3'-terminal bulged stem-loop was documented. (iii) Enhanced translation of a viral genomic RNA was demonstrated as a function of 2'-O-MT methylating activity. (iv) A requirement for 2'-O-MT methylating activity for virus replication was demonstrated with the use of the mouse hepatitis coronavirus reverse genetics system. (v) Over-expression of the viral 2'-O-MT in virus-infected cells had no detrimental *in-trans* effect on viral genome replication, transcription, or translation, but affected DI RNA accumulation *in-cis*. These results indicate that the coronaviral 2'-O-MT is essential for virus genome translation and replication and, as a multifunctional protein, is an attractive target for development of an anticoronaviral therapeutic drug.

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LIST OF ABBREVIATIONS

aa	amino acid
AMV	alfalfa mosaic virus
2'-O-MT	2'-O-methyltransferase
8mer-BSL	octamer associated bulge stem-loop
BCoV	bovine coronavirus
BHK	baby hamster kidney cells
BSL	bulged stem-loop
PK	pseudoknot
BSL-PK	bulged stem-loop/pseudoknot
CoV	coronavirus
DBT	delayed brain tumor cells
CIP	calf intestinal alkaline phosphatase
DI RNA	defective interfering RNA
EZH2	enhancer of zeste homolog 2 (Drosophila)
FCoV	feline coronavirus
FIP	feline infectious peritonitis
hnRNP	heterogeneous nuclear ribonucleoprotein
HCV	hepatitis C virus
HCoV	human coronavirus
hpi	hours post infection
HRT	human rectal tumor
HSP	heat shock protein
IBV	infectious bronchitis virus
kb	kilobase
kDa	kilodalton
MHV	mouse hepatitis coronavirus
MHVR	MHV receptor
Min	minute
MT	methyltransferase
N	nucleocapsid protein
N7MT	guanine-N7 methyltransferase
Ni-NTA	nickel-charged resin
NS5	nonstructural protein 5
Nsp	nonstructural protein
NSUN2	NOL1/NOP2/Sun domain family, member 2
nt	nucleotide
ORF	open reading frame
PABP	poly (A) binding protein
PCBP	poly (C) binding protein
PTB	polypyrimidine tract binding protein
PV	poliovirus

RdRp	RNA-dependent RNA-polymerase
RRL	rabbit reticulocyte lysate
RLuc	renilla luciferase
SAM	S-adenosyl-L-methionine
SARS	severe acute respiratory syndrome
SARS-CoV	SARS coronavirus
sgmRNA	subgenomic messenger RNA
SL	stem-loop
TBE	tris-borate-EDTA buffer
TGEV	transmissible gastroenteritis virus
UTR	untranslated region
VP1/ (2)	virus passage to fresh cells (indicates packaging of progeny viral RNA)
WGE	wheat germ extract
WNV	West Nile Virus
VP 39/55	viral protein 39/55

CHAPTER I. LITERATURE REVIEW

BACKGROUND

What are coronaviruses?

Coronaviruses (CoV), together with *toroviruses*, *arteriviruses*, and *roniviruses*, belong to the order *Nidovirales* of positive-strand RNA viruses [1]. At first isolated in the 1930s, new viruses in the CoV family are continuously being isolated from birds and mammals including humans. CoV are divided into three distinct groups: 1, 2 and 3, based on antigenic and genome sequence differences (Table 1) (all tables and figures are located in the Appendix F) [2] .

CoV infections are common in both birds and mammals. Resulting diseases are primarily of the respiratory and gastrointestinal tracts, some of which are associated with significant mortality in domestic pets and farm animals and are costly to the farming industry. Group 1 of CoV includes two important veterinary pathogens, the porcine transmissible gastroenteritis virus (TGEV) and the feline enteric coronavirus (FCoV). TGEV infection results in diarrhea in young animals with mortality rates reaching 60%. FCoV infection can result in feline infectious peritonitis (FIP), a disease associated with high mortality in cats [3]. Group 3 of CoV includes infectious bronchitis virus (IBV) and other avian CoVs that cause serious respiratory and enteric diseases in birds [4]. Group 2 of CoV include several pathogens of animals and humans that have been the most studied. These include the mouse hepatitis coronavirus (MHV) that has been the most studied as a model for CoV replication and pathogenesis. Some strains of MHV induce demyelinating encephalitis in mice and therefore this virus has been used as a model for human multiple sclerosis [5]. Other group 2 members that have been studied in detail include the bovine coronavirus (BCoV) and the recently discovered human SARS coronavirus (SARS-CoV).

BCoV is the primary etiological agent of severe diarrhea and mild respiratory infections

in newborn calves. In adult cattle BCoV infection is associated with winter dysentery (WD). BCoV infections are common in Europe, North America, Asia and New Zealand causing a serious treat to farming industry.

In humans, prior to SARS-CoV, CoVs were thought to account for ~25% of common colds in adults and occasional mild enteric infections. Recently, the outbreak of SARS-CoV induced a dramatic renewed interest in CoV replication and pathogenesis. The severe acute respiratory syndrome (SARS) epidemic resulted in over 8,000 infections with ~10% mortality rate worldwide (http://www.who.int/csr/sars/country/table2004_04_21/en/index.html). SARS-CoV has been shown to have a unique pathogenesis associated with severe pneumonia. Since the outbreak of SARS-CoV, over 200 new strains of CoV have been isolated from bats, birds, humans and other mammals including whales (www.ncbi.nlm.nih.gov) [6-8].

CoV replication.

BCoV, as all members in the family, is a positive-sense, single-stranded RNA virus with a 5' terminal methylated cap and 3' terminal poly (A) tail [9, 10]. The BCoV genome of 31,028 bases is the largest genome of any known RNA virus [11]. BCoV replicates in the cytoplasm of infected cells in a membrane-protected environment (Figure 1) [12]. After docking and entry, the virus particle is uncoated and genomic RNA is released and used as a template to generate viral replicase proteins by using the host cell translation machinery [13]. The replicase proteins are used to replicate the viral genome and to transcribe a 3' co-terminal nested set of capped and polyadenylated subgenomic mRNAs (sgmRNA) in a process called discontinuous transcription [14]. Each sgmRNA contains a common leader sequence of 65 nucleotides (nt) in length at its 5' terminus [15]. The CoV leader sequence makes up only part of the respective 5' UTRs, that for

BCoV range from 70-193 nt [16]. All sgmRNAs contain the genomic 289 nt long 3' UTR and poly (A) tail. In BCoV there are eight sgmRNAs (Figure 1) [15]. The sgmRNAs encode viral structural proteins and four accessory proteins of unknown functions. Only one protein is translated from each sgmRNA and this is from the 5' most open reading frame (ORF). The structural proteins assemble into the virion. After replication, genomic RNA is coated with N protein and packaged into virus particles by budding through the endoplasmic reticulum-early Golgi membranes [17, 18].

BCoV defective-interfering RNA (DI RNA).

Both the 5' and 3' UTRs of the CoV genome have been presumed to contain *cis*-acting elements required for genome replication. Most studies of BCoV *cis*-acting replication elements have utilized a naturally occurring 2.13 kilobase (kb) DI RNA as a model replicon due to the large size of the genomic RNA [19]. Since DI RNA molecules can replicate only in the presence of the helper virus, they must retain all the *cis*-acting signals necessary for genome replication and packaging of the viral genome [20]. The *trans*-acting factors, such as the viral genome encoded proteins that are necessary for DI RNA replication, are provided from the helper CoV (Figure 2). The naturally occurring BCoV DI RNA is composed of the fused termini of the viral genome. That is, the 5' terminal 496 nt (which includes the 210 nt 5' UTR and 285 nt of gene 1a) are fused with the 3' terminal 1637 nt (which includes the entire nucleocapsid (N) ORF sequence and the 3' UTR of 289 nt). In addition there is a poly (A) tail of 65 nt [21]. To facilitate replication studies, an in-frame 30 nt reporter sequence has been inserted into the N ORF (Figure 2). All characterized coronavirus DI RNAs contain the entire 5' and 3' UTRs and a portion of the first nonstructural protein (nsp1) coding region [22]. Several studies with the DI RNA of MHV

and BCoV have identified the *cis*-acting elements within the 5' UTR and 3' UTR of the CoV genome.

Cis-acting replication elements in the CoV genome.

With the use of DI RNA seven *cis*-acting elements in the 5' and 3' UTR have been identified and shown to function in MHV and BCoV DI RNA replication (Figure 2). Three *cis*-acting elements have been identified in the BCoV 5' UTR. The first was the leader sequence which was shown to function in DI RNA replication. Stem-loops (SL) I (nt 11 to 42) and II (nt 51 to 84) that were predicted to exist by the Tinoco algorithm and confirmed by structure probing are part of this element, but whether they, as higher-order structures, are part of the *cis*-acting signal is unknown [19, 23]. Just recently, two SLs, 1 and 2, in the same region (nt 1 to 51) were described in MHV and one of these (SL 2) has been shown to be a *cis*-acting replication element [24]. SLIII (nt 97 to 116) and IV (nt 186 to 215) have been confirmed to exist by RNase structure probing and to be required for DI RNA replication [25, 26]. Two additional *cis*-acting stem-loop structures SLV (nt 239 to 310) and SLVI (nt 311 to 340) were identified within the coding region of nsp1 [27, 28].

Four *cis*-acting elements have been found in the BCoV 3' UTR. They have been demonstrated to function as *cis*-acting signals in DI RNA replication and in some cases in viral genome replication. The first is a 5' proximal bulged stem-loop (BSL) that maps just downstream of the N stop codon (nt -289 to -222) [29]. The second is a hairpin-type pseudoknot (PK) that maps 63 nt downstream of the N stop codon (nt -226 to -173). BSL and PK were shown to share the same sequence [30]. This arrangement suggested that BSL and PK cannot exist simultaneously and thus the two structures were proposed to form one functional element

with alternative conformations. This larger structure was proposed to function as a molecular switch (BSL-PK) [31] but its function as a switch has not yet been demonstrated. The third is a 3' proximal octamer-associated bulged stem-loop (8mer-BSL) (nt -130 to -48) [32] that contains a conserved octamer sequence GGAAGAGC, present in essentially all CoVs sequenced to date (Figure 3). The three exceptions are a pigeon CoV (GGAGGAGC), a ferret enteric CoV (GGAAGGGC) and IBV UK/9/9/68 (GGGAGAGC) (<http://www.ncbi.nlm.nih.gov/sites/entrez>). The fourth *cis*-acting element is the 3' terminal poly (A) tail [21].

Although the described *cis*-acting higher-order elements have been shown to be required for DI RNA replication their exact function in genome replication remains to be determined.

CoV genome translation

CoV replication takes place in the cytoplasm and starts with the translation of the viral genome. Based on their structure, the 5' terminally-capped and 3' terminally-polyadenylated viral RNAs, both genomic and subgenomic, are believed to use the cap-dependent mechanism for initiation of translation and to follow the classical closed-loop model for enhancement of translation initiation via interactions between the 5' cap structure and 3' poly (A) tail [22]. Moreover it is presumed that the signals for translation regulation are localized at the viral 5' and 3' termini. After virus entry and uncoating, the genomic RNA undergoes translation which produces the viral proteins that are required for RNA synthesis and replication. The polycistronic genome encodes two large polyproteins pp1a and pp1ab which are products of ORF 1a and ORF 1ab, respectively (Figure 2). Pp1ab results from a -1 ribosomal frameshift at the ORF1a/1b junction [33]. Polyproteins undergo proteolytic processing by two virus-encoded proteases nsp3 and nsp5 [34, 35] into 16 nonstructural proteins (nsp). The 16 protein end products, possibly with

some of the intermediate precursors are believed to make up the viral RNA replicase-transcriptase complex. Putative functions have been assigned to four of the 11 predicted polyprotein cleavage products of ORF1a (nsp 1-11) and to all 5 proteins of the cleavage products of ORF1b (nsp 12-16). The ORF1b products are identified as an RNA-dependent RNA-polymerase (RdRp) (nsp12) [36], a helicase (Hel) (nsp13), an exonuclease (ExoN) (nsp14), an endonuclease (NendoU) (nsp15), and a 2'-O-methyltransferase (2'-O-MT) (nsp16) [11, 35]. Because of its large genome size, it is possible that CoV could have developed some replication strategies that differ from those of other RNA viruses. Several viral nonstructural proteins such as nsp 8 (RNA primase) [37], nsp14 [38], and nsp15 [39] are unique to CoV and not identified in other RNA virus families. Even though these proteins are apparent homologs of well known cellular RNA processing enzymes and are similar to some proteins that function in DNA virus replication, their function in CoV genome replication remains to be determined. In addition, nonstructural proteins analogous to replicase proteins of other RNA viruses or to cellular RNA processing enzymes, for example nsp12, nsp13 and nsp16, may have functional features unique to CoV.

CoVs make a 3' co-terminal nested set of capped and polyadenylated sgRNAs which encode the structural and accessory proteins. All but one of these, the shortest, is also structurally polycistronic but functionally monocistronic in the sense that only the 5' most ORF on the sgRNA is translated to make a protein (Figure 1). SgRNA-expressed proteins include the spike glycoprotein (S), the envelope protein (E), the membrane protein (M), and the nucleocapsid protein (N). Accessory proteins are often group specific. For example, the

hemagglutinin-esterase (HE) is found in only group 2 CoVs (Figure 2) [22]. The function of many of the accessory proteins remains to be determined.

OBSERVATIONS THAT LED TO THIS RESEACH

Structure of the 8mer-BSL in the BCoV 3' UTR.

The RNA *cis*-acting elements involved in the virus genome replication and transcription of the 3' terminal nested set of sgRNAs have so far been shown to localize at the viral UTRs and few have been found to be embedded within coding regions of the genome. Most analyses of coronaviral 3' UTR *cis*-acting elements have been done in DI RNAs of BCoV and MHV. Four *cis*-acting elements have been identified in the BCoV and MHV 3' UTR and shown to function in DI RNA replication. A majority of the analyses had been performed on the 3' UTR upstream structures which include the pseudoknot first described by our laboratory [29] and an adjacent bulged stem-loop first described by the P. Masters labolatory [31]. 3' UTR downstream structures had been studied for the most part in MHV. The structure of the downstream 3' terminal region in MHV had been predicted by the Tinoco algorithm and confirmed by enzyme structure probing [32]. No data had been available for corresponding region in BCoV. In this study the structure of the 3' terminal 162 nt of the BCoV genome, a region that contains a predicted 8mer-BSL structure and a presumed 45 nt long promoter for the negative-strand synthesis [40, 41], was determined with *mfold* prediction and enzymatic structure probing analysis.

Proteins interacting with *cis*-acting elements in the CoV genome.

The exact functions of the *cis*-acting replication elements in virus replication are still unknown. Based on precedents in other positive-strand RNA viruses such as poliovirus (PV) [42] Sindbis virus, a togavirus [43] and human hepatitis C virus (HCV), a flavivirus [44], it is postulated, that the *cis*-acting signals in UTRs function in replication by recruiting the necessary viral and cellular proteins. Since it has been established that both viral and cellular proteins interacting with the viral RNA structures play regulatory functions in genome replication, it becomes important to identify these for CoV. Until now, mostly cellular proteins have been identified that bind to both the 5' and 3' UTR structures in group 2 CoVs and almost all of these studies have been done with MHV. hnRNP A1 was identified to bind to the 5' UTR of MHV [45]. Moreover, hnRNP A1 was also found to bind the leader sequence and anti-leader sequence [45, 46]. The MHV 3' UTR was found to interact with mitochondrial aconitase [47], three mitochondrial heat shock proteins (HSP 40, HSP 60, HSP 70) [48], hnRNP A1 [46], PTB [49], and PABP [21]. Potential functions in genome replication were proposed for only hnRNP A1 and PTB. Based on the interaction of these proteins with the viral template and with each other, their function in genome circularization and regulation of genome replication was proposed [49]. Using BCoV and the BCoV DI RNA our laboratory has carried out many of the initial studies to define 5' and 3' proximal *cis*-acting RNA structures for CoV RNA replication. Additionally our laboratory has identified several cellular proteins binding to 5' UTR *cis*-acting elements. This study expands our analysis of the 3' UTR structures with the goal to identify cellular and viral proteins binding to the upstream 129 nt of the 3' UTR that contains the bulged stem-loop/

pseudoknot and the downstream 162 nt region containing predicted octamer-associated bulged stem-loop and the 45 nt promoter for minus-strand synthesis.

CoV encoded 2'-O-MT (nsp16).

The discovery of the SARS-CoV in 2003 increased NIH interest in the structure-function of CoV encoded proteins that make up the viral replication-transcription complex. This led to expression and characterization of the 16 individual cistrons encoded in BCoV ORF1. One of the first proteins expressed was nsp16, predicted by bioinformatics to be an *S*-adenosyl-*L*-methionine (SAM)-dependent RNA (nucleoside-2'-O)-methyltransferase (2'-O-MT) [35, 50]. The presumed function for viral 2'-O-MT is to methylate the 5' terminal cap structure on viral RNAs based on analogy to the homologous enzyme in flaviviruses [51]. The analysis of 2'-O-MT has been undertaken after its successful cloning and expression in *E. coli* and is presented in this dissertation.

The RNA 5' cap structure on eukaryotic mRNAs is a modified guanine nucleotide that is added to the 5' end of the cellular pre-mRNA using a 5'-5' triphosphate linkage. For many viruses that replicate in the cytoplasm it is added to 5' terminus of viral RNAs at the time of synthesis. The eukaryotic 5' cap stimulates the initiation of RNA translation since it enhances assembly of the pre-initiation complex [52]. Moreover, the cap protects RNA from degradation by 5'-3' exonucleases [53, 54]. It was also shown to be important for other processes such as nuclear export and splicing [55]. The cap-0 formation requires the subsequent function of 3 enzymes, (i) a 5' triphosphatase that removes the 5' γ -phosphate group of the RNA, (ii) a guanylyltransferase which adds GTP to the 5' terminus that results in the 5'-5' triphosphate linkage, and (iii) a guanine-N7-methyltransferase (N7MT) that methylates the 7-nitrogen of

guanine. In higher eukaryotes and certain viruses the cap-0 structure becomes modified into cap-1 or cap-2 structures with the help of 2'-O-MT that methylates the ribose at first or second transcribed RNA nucleotide, respectively. Since the capping process of cellular messages is restricted to the cell nucleus as a co-transcriptional mRNA modification, many intracytoplasmatically replicating viruses that possess cap structures have to encode their own capping machinery.

The CoV genome and sgRNAs are made in the cytoplasm and contain a 5' methylated cap and 3' terminal poly (A) tail. For MHV, the presence of a cap on viral RNAs was identified with two-dimensional oligonucleotide fingerprinting analyses by Lai et al [9]. However the CoV cap structure has not been determined to date. The capping mechanisms in the CoV have only recently been identified and have been reported concurrent with studies presented in this dissertation. Nsp13 was postulated to be involved in the capping reaction and to function as a 5' triphosphatase in addition to its helicase activity [56, 57]. Nsp14 was very recently found to function as N7MT [58]. The guanylyltransferase involved in the CoV capping reaction has not yet been identified.

The most C-terminal protein to be released proteolytically from pp1ab by nsp5-directed cleavage, nsp16, was predicted by bioinformatic analyses to be a 2'-O-MT. This prediction was based on (i) the presence of predicted structure of the so-called Rossmann fold [59], (ii) the presence of a structural similarity to the RrmJ family of methyltransferases [60], and (iii) the presence of the conserved methyltransferase catalytic tetrad K-D-K-E (Figure 16) [61]. The protein is conserved among CoVs and shows a 26.5% amino acid sequence identity and an 87.7% amino acid sequence similarity among the three CoV groups (Figure 4). Based on

precedents, the 2'-O-MT may be expected to be an RNA binding protein and bind genomic RNA. This property has been shown for members of the RrmJ family of MTs such as RrmJ/FtsJ which methylates 23 S rRNA in *E. coli* [62], VP39 in vaccinia virus [63] and NS5 in flaviviruses [51, 64]. It is known that MTs utilize a wide range of RNA binding motifs, most of which appear unique for each MT. This uniqueness presumably results from differences in substrate specificities [59]. In this study the analysis of the BCoV 2'-O-MT binding RNA targets was made including the expected binding to the 5'-terminal cap structure, the predicted site for 2'-O-MT enzymatic activity.

The BCoV 2'-O-MT has been predicted to function in the RNA capping process of the 5' end of viral RNA by analogy to the homologous enzyme in flaviviruses [51]. This function has been also reported for the group 1 FCoV 2'-O-MT [65]. Alternatively, it has been proposed that 2'-O-MT can methylate selected nucleosides in virus and/or cellular RNAs as part of a complex of unique CoV RNA-processing enzymes [35]. This is in agreement with reported RNA cleavage by nsp15 which is inhibited by 2'-O-MT base methylation [66]. Moreover the analysis of MHV temperature-sensitive mutants suggested the potential for a direct function of 2'-O-MT in RNA synthesis or RNA stabilization [67]. At this time the exact function of 2'-O-MT in CoV replication is unknown. In this study the characterization of the 2'-O-MT function in the viral genome replication is presented including analysis of the 2'-O-MT predicted function in translation of cognate viral messages. Additionally since 2'-O-MT is presumed to function as part of the viral replicase-transcriptase complex the 2'-O-MT interactions with other viral proteins were analyzed that implicate its role in formation of a 3' terminal protein complex.

**CHAPTER II. BOVINE CORONAVIRUS 2'-O METHYLTRANSFERASE (NSP 16)
BINDS A 3' TERMINAL GENOMIC CIS REPLICATION STEM-LOOP.¹**

¹ This chapter will be submitted for publication in the *Journal of Virology*. “Bovine Coronavirus 2'-O-Methyltransferase (nsp 16) Binds a 3' Terminal Genomic Cis Replication Stem-Loop” Agnieszka Dziduszko ², Kortney M. Gustin ¹, Tara Tucker ¹ and David A. Brian* ^{1, 2}, Departments of Microbiology ¹ and Pathobiology ², University of Tennessee College of Veterinary Medicine, Knoxville, Tennessee 37996-0845. *Corresponding Author. Work performed by coauthor Tara Tucker includes cloning of 2'-O-MT into pGEX vector, for antibody generation. Gel-shift experiments with the 5' UTR structures SL IV, SL V and SL VI and 2'-O-MT were performed by coauthor Kortney M Gustin. Gel shift experiment with SLIII and 2'-O-MT was performed by Bo-Jhih Guan.

INTRODUCTION

Coronaviruses are enveloped, single-stranded, positive-sense RNA viruses, with the largest known RNA genome (~30 kb) [11]. Viral replication takes place in the cytoplasm and the genome serves as a translation template for synthesis of the replicase proteins, and as a transcription template for its own replication and the synthesis of negative-strand templates used for generating a 3' coterminal nested set of subgenomic (sg) mRNAs. By virtue of a common leader (65 to 93 nt, depending on the coronavirus species), the genome and sg mRNAs are also 5' coterminal and contain a 5' methylated cap. Synthesis of the coronaviral replicase proteins occurs immediately after viral entry and involves translation of the 5' two-thirds of the genome which constitutes gene 1. Gene 1 open reading frame (ORF 1) is translated to produce two polyproteins, pp1a and pp1ab, which undergo co- or post-translational proteolytical processing into 16 nonstructural proteins named nsp 1-16. Pp1ab results from a -1 ribosomal frameshift at the ORF1a/1b junction [33] and proteolytical processing is carried out by two ORF 1a-encoded viral proteases, nsp 3, a papain-like protease, and nsp 5, the main protease [34]. It has been postulated that the 16 products of gene 1, either as partially or completely proteolytically processed proteins, function together as the replicase-transcriptase protein complex for RNA synthesis. Precisely how each functions and whether all are components of a replicase-transcriptase complex is not yet fully known.

Perhaps as a consequence of their large genome size, coronaviruses possess unique features for RNA replication and transcription. The nonstructural proteins encoded by gene 1 identified as a primase (nsp 8) [37], ExoN (nsp 15) [38] and XendoU (nsp 14) [39], for example, are unique to the coronaviridae. In addition, gene 1-encoded nonstructural proteins appearing

analogous to replicase proteins of other RNA viruses are the RNA-dependent RNA polymerase (RdRp) (nsp 12), the helicase (nsp 13) and the 2'-O-MT (nsp 16).

The most C-terminal replicase protein (nsp 16) has been predicted by bioinformatics to be a 2'-O-MT [35, 50]. This prediction was based on (i) the presence of the so-called Rossmann fold, (ii) a structural similarity to the RrmJ family of methyltransferases, and (iii) the presence of the conserved catalytic tetrad motif, K-D-K-E. These features are found in the 2'-O-MTs of all three subgroups of coronaviruses which share an aa sequence identity of ~26.5% and ~87.7% sequence similarity (Figure 4). The 2'-O-MT activity for nsp 16 of the feline coronavirus has been demonstrated recently by Decroly et al [65] to methylate short RNAs containing a cap-0 structure ($^7\text{mGpppAC}_{3-6}$ in the feline coronavirus). The N-methylating function may be carried out by a second recently identified enzyme activity of nsp 14 (NendoU) [58]. Our work on the 2'-O-MT of BCoV began with the goals of demonstrating its (i) cap methylating activity and (ii) determining its potential to bind the 5' cap-containing *cis*-acting RNA targets and other *cis*-acting RNA elements identified for BCoV and MHV. The second goal was based on the precedent that members of the RrmJ family of methyltransferases, including RrmJ/FtsJ which methylates 23 S rRNA in *E. coli* [60-62], VP39 which methylates viral RNA in vaccinia virus and is a co-factor for 3' terminal polyadenylation [63], and the flavivirus 2'-O-MT which methylates viral genomic RNA [51, 64], show RNA binding with a wide range of targets and are in some cases multifunctional. Although methylating activity was not conclusively found for reasons described below, an unpredicted binding target was identified.

Here we report that in conformity with conditions for binding and methylation of an N7-methylated type 0 cap ($^7\text{mGpppAC}_{3-6}$) by the feline coronavirus 2'-O-MT [65], a type 0 cap

(^{7m}GpppGA₃₀ in the case of BCoV) and no tRNA in the binding buffer are required for 5' proximal binding by the BCoV 2'-O-MT. We also report a surprising specific, high affinity (~50 nM) binding to the 3' terminal 162 nt region of the 289 nt BCoV 3' UTR. The 3' terminal region of the genome contains the 3' terminal octamer-associated bulged stem-loop and the 3' terminal 45 nt, which is a documented *cis*-replication element [40, 68]. Binding appeared dependent on a platform formed by secondary and perhaps tertiary RNA interactions. We postulate, furthermore, that the interaction between the 2'-O-MT and 3' end is conserved among coronaviruses since the BCoV 2'-O-MT also binds the homologous structure in SARS-CoV. We propose that in addition to its role in methylating the 5' cap, the 2'-O-MT functions at the 3' terminus, possibly in the assembly of the replication-transcription complex.

MATERIALS AND METHODS

GenBank accession numbers.

GenBank accession numbers are U00735 for BCoV-Mebus strain and NC_004718 for SARS-CoV-Tor2 isolate.

Virus and cells.

The Mebus strain of BCoV was grown on human rectal tumor (HRT-18) [69] cells as described [23].

cDNA cloning of BCoV 2'-O-MT into expression vectors.

Standard cDNA cloning methods were used to clone the BCoV 2'-O-MT gene into two expression vectors. In the first, the 2'-O-MT ORF (genome nt 20,595-21,494) was cDNA cloned into the BamH I and EcoR I sites of the pGEX vector (Invitrogen) (pGEX-GST-BCoV-2'-O-

MT). In the second, the 2'-O-MT ORF was subcloned into Champion pET-6xHis-SUMO vector using T/A ligase technology (Invitrogen) (pET-6xHis-SUMO-BCoV-2'-O-MT).

Preparation of antibody against BCoV 2'-O-MT.

GST-BCoV-2'-O-MT in the pGEX vector was expressed in *E. coli* and used to prepare antiserum in rabbits by Proteintech Group Inc. (<http://www.ptglab.com/>).

Preparation of BCoV 2'-O-MT for testing enzyme and binding activities.

For initial enzyme and binding studies, GST-BCoV-2'-O-MT purified from pGEX-GST-BCoV-2'-O-MT by Proteintech Group Inc. was used.

For detailed binding studies, tag-free BCoV-2'-O-MT was purified from pET-6xHis-SUMO-BCoV-2'-O-MT. PET-6xHis-SUMO-BCoV-2'-O-MT vector was maintained in One Shot-Mach1-T1 *E. coli*. Freshly transformed into BL21 (DE3) *E. coli* was grown at 20° C for 12 h in LB medium containing 25 µg/ml kanamycin and protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) final concentration. Cells at ~0.6-0.7 OD₆₀₀ were pelleted, resuspended in lysis buffer (3ml/1g of bacteria pellet) (1xPBS containing 200 mM NaCl, 10 mM imidazol, 5% glycerol, 1% NP-40, 1 mM βME, 1 mM PMSF (phenylmethanesulphonylfluoride), and 1xEDTA-free Complete Protease Inhibitor (Roche)), and lysed on ice by sonication in the presence of lysozyme (1mg/ml) (Biomedicals) and Benzonase endonuclease (Novagen) (250 units/5g of bacteria pellet). Fusion protein was purified from the soluble fraction by chromatography on Ni-NTA superflow resin (Qiagen). 6xHis-SUMO-2'-O-MT was eluted with elution buffer (1xPBS containing 200 mM NaCl, 300 mM imidazole, 5% glycerol, 1% NP-40, 1 mM DTT). SUMO protease was added and the cleavage reaction carried out for 2 h at 37° C. SUMO protease-cleaved 6xHis-SUMO tag was re-bound to Ni-NTA resin

and tag-free ~33.6 kDa 2'-O-MT recovered. Protein concentrations were determined by the Bradford assay and protein purity was evaluated by SDS-PAGE on a 12% polyacrylamide gel.

Synthesis of RNA probes.

PCR amplified DNAs of *cis*-replication stem-loops from cDNA clones of BCoV DI RNA mapping within the 5' and 3' terminal regions of the BCoV genome were cloned into pGEM3Zf (-) (Promega) and probes were generated with T7 RNA polymerase. These were the 5' proximal 30nt (5'30nt) (nt 1-30) probe and 5' proximal 120 nt SLI-II probe (nt 1-120) [23], the 115 nt SLIII probe (nt 68-182) [25], the 109 nt SLIV probe (nt 153-261) [26], the 134 nt SLV-VI probe (nt 231-364) [27, 28], and, counting bases from the 3' end, the 3' proximal 129 nt bulged stem-loop/pseudoknot (BSL-PK) probe (nt 162-291) [29], and the 162 nt octamer-associated bulged stem-loop (8mer-BSL) probe (nt 1-162). Probes representing truncated or mutated 3' terminal structures were likewise prepared. These were the 63 nt 8mer-BSL-left probe (nt 100-162), the 100 nt 8mer-BSL-right probe (nt 1-100), the 8mer-BSL48-147 probe (nt 48-147), the 8mer-BSL70-125 probe (nt 70-125), the 8mer-BSL85-112 probe (nt 85-112), and the 8mer-BSL1-55 probe (nt 1-55). The 3' terminal 8mer-BSL-SARS probe (nt 1-213) was likewise prepared. For PCR amplification the proofreading polymerase AccuPrime TaqHIFI (Invitrogen) was used and PCR products were digested with EcoR I and Hind III and purified with the QIAEX II gel extraction kit (QIAGEN). The 5'30nt, SLI-II, 8mer-BSL85-112, and 8mer-BSL1-55 probes were cloned with the T7 RNA polymerase promoter into the EcoRI/HindIII site of pUC19. Site-directed mutagenesis was used to generate mutants of the 8mer-BSL which includes the octamer mutant (oct. mut.), the loop mutant (loop mut.), and the octamer deletion mutant (oct.del.). All mutations were confirmed by sequencing.

Gel shift assay.

The gel shift assay was used as described by Andino et al. [42] except where noted. Briefly, constructs in pGEM3Zf (-) and pUC19 were linearized with Hind III and uniformly labeled probes were synthesized with T7 RNA polymerase in the presence of [α - ^{32} P] UTP (120 μ Ci), 100 μ M UTP [26] and purified by electrophoresis on gels of 6% polyacrylamide-7 M urea. Probe at $\sim 2\text{--}6 \times 10^4$ cpm and 1-2 ng was incubated with the indicated concentrations of 2'-O-MT for 10 min at 37 °C in binding buffer (5 mM HEPES, 40 mM KCl, 2 mM MgCl₂, 2 mM DTT, 4% glycerol, 1xEDTA-free Proteinase Inhibitor Complete (Roche), 0.5 unit/ μ l RNasin (40 μ g/ μ l) (Promega), 0.25 μ g/ μ l heparin, 1 μ g/ μ l tRNA). In some preparations as noted, tRNA was omitted. RNA-protein interactions were analyzed by nondenaturing 6% PAGE at 100 V for 4 hr at 4 °C and autoradiography. Where indicated, unlabeled probe (as specific competitor) and RNAs representing other regions of the genome, tRNA, polyA:U, or heparin (as presumed non-specific competitors) were included in the pre-incubation step of the binding reaction. For kinetic analyses of complex formation the amount of radioactivity in the protein-RNA complex was quantified with a Phosphoimager. Apparent K_d was estimated from the protein concentration at which 50% of available labeled RNA appeared in the complex.

Computer prediction of RNA secondary structure.

RNA secondary structure predictions of the 3' terminal 162 nt of the BCoV genome without a poly (A) tail, and of the 3' terminal 162 nt but with a 5' terminal vector-derived GGGCGAAUUC sequence and a 3' terminal vector derived A but no poly (A) tail were made with *Mfold* version 3.2 (<http://frontend.bioinfo.rpi.edu/applications/mfold/>) (Figure 8) [70, 71].

Enzyme structure probing of the 3' terminal 162 nt.

Enzyme structure probing was carried out essentially as described by Henriët et al [72]. Briefly, 8mer-BSL RNA was generated with the RiboMAX large scale RNA production System-T7 (Promega). *In-vitro* synthesized RNA contained a 5' terminal 10 nt vector-derived sequence and the 3' terminal 162 nt 8mer-BSL sequence and a vector-derived 3' terminal A. RNA (~20ug) was dephosphorylated with calf intestinal alkaline phosphatase (CIP, 10 U/μl; NEB) and 5' end-labeled with 25 pmol [γ - ^{32} P] ATP (7000 Ci/mmol, 150 mCi/ml [ICN]) and T4 Polynucleotide Kinase (10 U/ml; NEB). Radiolabeled RNA was purified by electrophoresis on a 6% PAGE/7M urea gel. Radiolabeled RNA (6×10^4 - 1×10^6 cpm, ~0.4 μM) was digested with the indicated concentrations of RNase T2 (Invitrogen), RNase T1 (Ambion), or RNase A (Ambion) for 15 min, or CV1 (Ambion) for 30 min. Reactions were terminated with inactivation/precipitation buffer (Ambion) and the partially digested RNA components were resolved by electrophoresis on a 6% polyacrylamide-8M urea gel for 2-3 hr at 1300 V and analyzed by autoradiography. A nucleotide ladder was generated by hydrolysis of the tested RNA in 1×Alkaline Hydrolysis Buffer (Ambion), for 5 min at 95 °C.

RNase footprinting of 2'-O-MT bound to the 3' terminal 8mer-BSL.

The protection of nucleotides from nuclease digestion in the presence of 2'-O-MT was carried out as described for enzyme structure probing but with the addition of the tested protein. For this, labeled RNA (6×10^4 - 1×10^6 cpm, ~0.4 μM) was used in 40 μl binding reactions with GST or GST-BCoV-2'-O-MT as described for the gel shift assay. Following binding, the reaction mixes were digested with the indicated concentrations of RNase T2 (Invitrogen), RNase T1 (Ambion), or RNase A (Ambion) for 15 min, or CV1 (Ambion) for 30 min, and reactions

were terminated with inactivation/precipitation buffer (Ambion). Pellets were resuspended in Acrylamide Gel Loading Buffer (Ambion) and the amount of precipitated radiolabeled RNA in each sample was determined by scintillation counting. RNA samples were resolved by electrophoresis as described for RNA structure probing.

DI RNA based *in-vivo* 2'-O-MT over-expression system.

The cDNA-cloned BCoV defective interfering RNA, pDrep12.7, a template used to express protein from a subgenomic mRNA, has been described [73]. The 2'-O-MT cistron with a C-terminal 3xGlycine linker (3xGly) and 6xHistidine Tag (6xHis) was cloned into the N protein coding region with an 18 nt intergenic sequence (IG) and flanking sequence at the 5' terminus that allows for synthesis of a sgRNA. For expression, DI RNA was transfected into HRT cells together with BCoV helper virus as previously described [23]. At 24 hr post-transfection cells in 25 cm² flasks (2.3 x 10⁶) were washed with ice cold 1xPBS and lysed for 20 min on ice with modified RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1x proteinase inhibitor complete (Roche), 1 mM EDTA, 10 mM imidazole). The lysate was clarified by centrifugation at 14,000 rpm at 4 °C for 10 min and treated with Micrococcal nuclease (160 U/ ml, Pharmacia) for 50 min/ 37 °C before inactivation with 5 mM EGTA for 10 min/ 37 °C. 6xHis-2'-O-MT in the lysate was bound to Ni-NTA resin for 30 min, followed by three washes with RIPA buffer with increasing NaCl concentrations to 0.45 mM. Bound 6xHis-2'-O-MT was eluted with RIPA buffer containing 300 mM imidazole.

Biotin-RNA pull-down of *in-vivo* over-expressed 2'-O-MT.

RiboMAX-generated 8mer-BSL RNA (Ribomax) was poly (A)-tailed [74] with BIO-11-ATP (PerkinElmer) (1 μM 8mer-BSL RNA, 5 mM ATP, 5 mM BIO-11-ATP, 10xPAP (Poly (A)

polymerase buffer) and 1 μ l PAP in 50 μ l reaction at 37 °C for 1 hr. The 8mer-BSL-BIOTIN poly (A)-tailed RNA was incubated with the *in-vivo* expressed 2'-O-MT preparation in binding buffer (RIPA buffer with 1 mM DTT, 10% glycerol, 20 U RNasin Inhibitor (Promega)) for 2 min at 37 °C, then for 30 min at 4 °C, at which time 30 μ l of avidin pre-blocked streptavidin bead suspension was added and incubation continued for 5 min at 20 °C. Beads were loaded onto centrifugal filter units (Millipore, 0.45 μ m) and washed three times with binding buffer with increased NaCl concentration up to 0.45 mM, and 2'-O-MT was eluted with binding buffer containing 1 M NaCl. Western Assay with 2'-O-MT specific rabbit polyclonal antibodies was used to identify the binding of *in-vivo* expressed 2'-O-MT to the tested RNA.

Western Analysis of biotin-RNA pull-down protein.

Biotin-RNA pull-down elution samples were subjected to 12% SDS polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane (Pierce) for Western blot analysis. Briefly, the membrane was blocked with 1:5 dilution of Super Block Blocking Buffer (Pierce) and then exposed to a rabbit 2'-OMT specific polyclonal antibody (1: 2000) (Proteintech Group Inc.) diluted in Super Block Blocking Buffer (1:5). After incubation with Goat anti-Rabbit IgG (H+L) antibody coupled with horseradish peroxidase (1: 30000 dilution) (Pierce), the signal was detected with Super Signal West Pico Substrate (Pierce).

RESULTS

2'-O-MT binds the 5' terminal ^{7m}G cap structure.

Our initial experiments were designed to demonstrate methylation of 5' terminal cap structure and 5' terminal 30 nt and 120 nt regions of the genomic RNA with purified 2'-O-MT

following a protocol used for flavivirus assays [51]. BCoV 2'-O-MT (Figure 5.C) expressed and purified from pET-SUMO vector was used. Neither cap methylation (data not shown) or binding (Figure 6.B for capped probe SLI-II) were found. With the recent report that the feline coronavirus 2'-O-methylation requires a ^{7m}G cap structure (^{7m}GpppAC₃₋₆ for feline coronavirus) for both binding and methylation, we reinvestigated BCoV 2'-O-MT binding properties on transcripts with a cap-0 structure for BCoV (^{7m}GpppGA₃₀). For this we used gels shift assay with slightly modified binding buffer, in which the non-specific competitor (tRNA) was omitted. The analysis revealed weak binding of 2'-O-MT under these conditions to ^{7m}GpppG capped 5'30nt RNA probe (Figure 6.C), but neither to GpppG-capped RNA nor uncapped RNA (data not shown).

BCoV 2'-O-MT binds a 3' terminal 162 nt *cis*-acting bulged stem-loop in the 3' UTR with specificity and moderately high affinity.

Based on the precedent that members of the RfmJ family of 2'-O-MTs are multifunctional and have varied RNA binding properties, we systematically investigated the binding of BCoV 2'-O-MT to six known *cis*-acting replication elements (Figure 6.A). These are located in the 5' UTR and adjacent ORF 1 region, and in the 3' UTR. Whereas binding to 5' SLI-II transcripts was found only when tRNA was omitted from the binding buffer (as described above), no binding was found to the 5' proximal SLIII, SLVI or SLV-VI, or to the 3' proximal *cis*-acting bulged stem-loop/pseudonknot structure (BSL-PK) under the conditions described by Andino et al (Figure 6.B). Surprisingly binding was found to the 3' terminal predicted 8mer-BSL region. It is noteworthy that the ³²P-labeled 8mer-BSL probe migrated as upper and lower forms of which only the upper form appeared quantitatively shifted by the 2'-O-MT (Figure

6.B). We interpret this to mean there are two differently folded probe populations and since only the upper band is totally shifted we assume this is the properly folded form. Nearly 50% of the upper band is shifted with 50 nM 2'-O-MT (Figure 6.B).

To determine whether binding of 2'-O-MT to the 8mer-BSL is specific, competition assays were carried out. Radiolabeled probe was incubated with 2'-O-MT in the presence of excess unlabeled potential competitor RNAs (Figure 7). The results show that complex formation was inhibited by unlabeled 8mer-BSL RNA, but not by tRNA, poly A:U, heparin, or unlabeled SLV-VI or BSL-PK.

These results together indicate that the BCoV 2'-O-MT specifically binds the 3' terminal 8mer-BSL with relatively high affinity.

BCoV 2'-O-MT binds truncated and 3' terminal regions of the 8mer-BSL but optimal binding requires an intact higher-order structure.

To locate the 2'-O-MT binding site(s) within the bulged stem-loop-containing 3' terminal region, truncated regions of the *mfold*-predicted 8mer-BSL structure (Figure 8.A) were used as T7 RNA polymerase-generated radiolabeled probes in gel shift assays. As can be seen in Figure 8.B, binding occurred with large downstream fragments (8mer-BSL-right) but not with extensively truncated upstream or downstream regions (i.e., 8mer-BSL-left, 8mer-BSL48-147, 8mer-BSL70-125, 8mer-BSL85-112 (data for 8mer-BSL70-125 is not shown), and 8mer-BSL1-55). These data together suggest that 2'-O-MT binding to the 8mer-BSL region is dependent on the overall higher-order RNA structure and requires the full length probe for highest binding affinity.

To determine if the predicted large, internal octamer-containing loop is involved in 2'-O-MT binding, nt 113-124 within the 8mer-BSL were mutated to convert the loop to a predicted double-stranded region (Figure 8.A) and the probe was tested for 2'-O-MT binding (Figure 8.B). Relative to full-length wt probe, binding to the loop mutant (loop mut.) was diminished but not lost entirely indicating that the apparent internal loop is not an absolute requirement for binding. Likewise, mutations changing the octamer sequence but predicted to retain the loop, i.e., GGAAGAGC→GGuucucC in "oct. mut." (Figure 8.A), led to diminished binding (Figure 8.B) indicating the wt octamer sequence is not an absolute requirement for binding. Similarly, deletion of the GGAAGAGC octamer sequence which converted the predicted loop 2 to a bulge and a small internal loop (Figure 8.A) only slightly reduced 2'-O-MT affinity (Figure 8.B). Based on these data we conclude that binding of 2'-O-MT to the 8mer-BSL is RNA structure dependent and that the octamer sequence and the large octamer-containing loop contribute to binding but are not required.

Secondary structure mapping of the 3' terminal 162 nt of the BCoV genome.

Mfold of the 3' terminal 162 nt of the BCoV 3' UTR, the region downstream of the characterized *cis*-acting BSL-PK, predicted an octamer-associated bulged stem-loop (8mer-BSL) within the 3' terminal 148 nt with the octamer GGAAGAGC positioned on the downstream side of a large internal loop as the most stable folded structure (Figure 9.B). A higher-order structure of the 8mer-BSL has been predicted similarly and enzymatically structure-mapped for MHV, a close relative of BCoV [32]. Curiously, a helical region of 8 nt is found adjacent to the octamer-associated internal loop (boxed in Figure 9.B). To characterize the 2'-O-MT binding sites on the 3' terminal BCoV genomic sequence, enzyme structure probing was first done on a 162 nt 3'

terminal sequence to test its higher-order structure against that predicted by *mfold*. For this, the method of Henriët et al [72] was used on a 5' ³²P-end labeled 173 nt RNA transcript containing 10 nt from the pGEM vector at 5' terminus and 162 nt from the poly (A)-tailless BCoV 3' terminus followed by vector derived A nt at 3' terminus. Aliquots of the transcript were treated with a single concentration of RNase A which cleaves at Cs and Us, and differing concentrations of the single-strand-specific RNase T2 which cleaves mostly at As, and the double-strand-specific RNase CV1, and the products were separated on a 8% polyacrylamide sequencing gel (Figure 9.A). From these results and the results shown in Figure 10.B of digestion with single concentrations of RNases A, T2 and CV1, and T1 which cleaves at Gs, the structure for approximately 126 nt from the 5' terminus was deduced (Figure 9.B). The probing results were largely consistent with the *mfold*-predicted bulged stem-loop structure although notable differences were observed and both are summarized from Figure 9.A and Figure 10.B as follows:

- (i) For loop 1, mostly strong single-strand (ss) hits were found for nt 98-104 indicating L1 is probably a terminal 9 nt loop rather than a tetraloop as predicted.
- (ii) For the 7 nt stem 2 with an internal bulge of 2As, largely strong ds hits on both sides show it to be completely double-stranded as predicted.
- (iii) For the large octamer-associated internal loop 2, surprising susceptibility to both ss RNases and ds CV1 were found. The presence of partially cleaved patterns by both RNases T2 and CV1 suggest the loop may exist in two alternative conformations, an open structure cleaved by RNaseT2 and a helically twisted conformation with stacked bases cleaved by RNaseCV1.
- (iv) For stem 2 which is found in close proximity to the octamer-associated L2, RNase cleavage was largely ds-specific indicating it is probably a stable stem.
- (v) For loop 3, only strong ss hits were found indicating this is a stable internal loop.
- (vi)

For stem 3, the upper 5 nt were susceptible to both ss and ds RNAses indicating this part of the stem may breathe and in so doing enlarges L3. The lower part of S3 behaved as stably double-stranded. (vii) For loop 4, both ss and ds hits were found throughout indicating bases in this loop may become stacked. (viii) Surprisingly, stem 4 showed strong properties of single- and double-strandedness that strongly suggest a breathing structure.

RNA footprinting of the 8mer-BSL reveals three sites of RNA protection and five sites of 2'-O-MT-induced RNA hypersensitivity.

Because of the apparent complexity of 2'-O-MT binding with 8mer-BSL RNA, RNA footprinting was done with 8mer-BSL and the oct. mut. for higher resolution of the binding sites. For this, RNase probing was done as described above in both the absence and presence of 2'-O-MT [72] (Figure 10). In the presence of 2'-O-MT, four sites of RNase protection were found. The first and strongest appeared in the terminal loop 1, identified by an open crescent in Figure 10.D. From Figure 10.B it can be seen for both RNases A and T1 that the amount of U101 and adjacent G cleaved is two-fold less in the presence of 2'-O-MT indicating protection. For U101, an increase in protection with increasing amounts of 2'-O-MT can be observed in Figure 10.C, which by scintillation counting of the excised gel bands, represents a 90% decrease in RNase susceptibility of RNA if 1:90 molar ratio of RNA to protein was used. Curiously, protection was observed against T2 RNase in the wt 8mer-BSL RNA (Figure 10.A), but not with octamer mutant RNA (Figure 10.B). A second site of protection localized at the loop 2 nt 118 just opposite of conserved octamer sequence, identified by the dashed crescent. A third site of protection that appeared somewhat weaker, identified by a dashed empty crescent in Figure 10.B, was for the octamer sequence itself (nt 71-78) where protection was observed against RNases

T2, T1, and CV1. No Us are present within this sequence so protection against RNase A cannot be ascertained. A fourth site of protection appeared broad ranging from nt U4 through U22 (Figure 10.B). Protection against both ss and ds-specific RNases were seen consistent with this region appearing as both ss and ds structures. It therefore appears that the 2'-O-MT binding site, per se, is a composite of several sites brought together by RNA secondary and perhaps tertiary interactions.

Curiously, five sites become hypersensitive to RNase treatment upon 2'-O-MT binding. Most striking is the hypersensitivity of the loop 2 region (nt 119-124) opposite the octamer sequence (nt 71-78) which shows increased cleavage by RNases T2, CV1, and A in Figure 10.B, lanes 4, 6 and 10, respectively, and by RNase T1 as well for the octamer mutant in Figure 10.A, lane 4, 6, 8. Other sites of hypersensitivity include a 6 nt region (nt 149-153) on the upstream side of L4 that show increase sensitivity to RNases T2 and CV1, a 3 nt region (nt 102-104) of L1 which show increased sensitivity to RNase CV1 (Figure 10.B), a 5 nt region (nt 43-47) on the downstream side of L4 (Figure 10.B), and a 3 nt region (nt 21-23) which shows increased sensitivity to RNases T2 and CV1 (Figure 10.B). Although it is not possible to discern what specific binding interactions cause the hypersensitivity, it is apparent that several disparate sites in the 8mer-BSL become structurally altered as a result of 2'-O-MT binding.

Biotin-RNA pull-down of *in-vivo* expressed 2'-O-MT.

To determine if 2'-O-MT expressed in the cytoplasm of BCoV-infected cells also binds the 8mer-BSL RNA, thus excluding the possibility that *E. coli*-expressed 2'-O-MT artificially binds 8mer-BSL because of lack of proper posttranslational protein modification(s), a pull-down assay with biotinylated 8mer-BSL RNA was employed. For this, a BCoV DI RNA-based 6xHis-

2'-O-MT expression system was used (described in details in Chapter IV). A characterized BCoV DI RNA (pDrep12.7) was modified to express 6xHis-2'-O-MT from an internal transcription signal (Figure 11). As shown in Figure 19, this system generates protein level easily detectable by Western assay with BCoV 2'-O-MT specific antibodies that is only weakly observed by BCoV infection alone. Thus, there appears on the basis of Western blotting to be a ~60-fold higher expression of the 2'-O-MT from DI RNA-based expression system than from BCoV-infected cells alone. To determine whether 6xHis-2'-O-MT would bind the 8mer-BSL, purified 8mer-BSL RNA with biotinylated poly (A) tail was used in a pull-down assay. The rationale for biotinylating only the poly (A) tail was to reduce the risk of improper RNA folding while at the same time providing efficient attachment of the RNA target to streptavidin. When 6xHis-2'-O-MT from DI RNA-transfected cell lysates was incubated with biotinylated 8mer-BSL RNA and purified on streptavidin beads, a relatively small amount of 6xHis-2'-O-MT was obtained relative to that present in the cell lysate (Figure 11). The low yield could mean that only part of the 8mer-BSL RNA population is in the proper conformation for binding as was observed for gel-shift assays, or that some of the 6xHis-2'-O-MT molecules are already assembled in replication complexes *in-vivo*, or in membranous compartments, and thus protected from micrococcal nuclease treatment designed to release them and unavailable for binding with synthetic RNA templates [75]. Additionally, some 6xHis-2'-O-MT may have been washed from the columns during purification. These data show that *in-vivo* over-expressed 6xHis-2'-O-MT likely binds the 8mer-BSL RNA within the cell which is consistent with the binding properties of 2'-O-MT synthesized in *E. coli* and tested *in-vitro*.

BCoV 2'-O-MT binds the SARS-CoV 8mer-BSL RNA.

To determine whether the binding of the 8mer-BSL by BCoV 2'-O-MT is a conserved feature among closely related CoVs, the *E. coli*-expressed BCoV 2'-O-MT was used in a gel shift assay with the SARS-CoV 8mer-BSL RNA, a region represented by the 3' most 194 nt of the SARS-CoV genome. This region is predicted by *mfold* to form a 8mer-BSL-like structure (Figure 12.A). A gel-shift analysis shows that the BCoV 2'-O-MT binds the SARS-CoV 8mer-BSL with high affinity similar to that of the BCoV 8mer-BSL (Figure 12.B).

DISCUSSION

In this study we present evidence showing the BCoV 2'-O-MT (nsp 16) to be an RNA binding protein that binds two sites in the genomic UTRs of BCoV. (i) In agreement with the recent report by Decroly et al, we found that 2'-O-MT expressed and purified from *E. coli* binds the 5' terminus which contains a type 0 methylated cap ($^7\text{mGpppGA}_{30}$ for BCoV) but under conditions which contain no tRNA in the binding buffer. In the presence of tRNA we found no binding at this site. (ii) 2'-O-MT binds with specificity and high affinity (estimated to be ~50 nM) to the 3' terminal region, nt 1-162 from the 3' end, which harbors the octamer-associated bulged stem-loop. It did not show binding under these conditions to other identified BCoV *cis*-replication structures which include the 5' proximal stem-loops I and II, III, and IV in the genomic 5' UTR, and V and VI in ORF1, and the 3' proximal BSL-PK in the 3' UTR. We further showed that binding to the 3' terminal 8mer-BSL was dependent on its higher-order structure and that footprinting analyses showed four sites of 2'-O-MT binding and five sites of nuclease hypersensitivity resulting from protein binding indicating that binding likely cause

alteration of its higher-order structure with extensive alteration of the octamer-associated internal loop region (L2). Binding to 8mer-BSL was also demonstrated with BCoV 2'-O-MT over-expressed in cells by biotinylated 8mer-BSL RNA pull-down assays. In addition, the BCoV 2'-O-MT was shown in a gel shift assay to bind the 3' terminal 8mer-BSL region of the distantly related group 2 SARS-CoV.

On the face of it, the binding of the BCoV 2'-O-MT to a 3' terminal conserved structure of group 2 CoVs that appears to be much stronger than to the 5' terminal cap structure, would suggest that it plays a role in events at the 3' end of the genome, or of the subgenomic mRNAs. It is possible that 2'-O-MT is multifunctional protein and, in addition to its cap methylating function, it might interact with RNA and other viral proteins at the 3' end to assemble the replication-transcription complex and take part in the initiation of negative-strand synthesis. This possibility, however, is largely difficult to reconcile with the results of studies by the Masters laboratory in which it was shown that a major portion of the 8mer-BSL (nt 46-156 in the MHV 3' UTR, equivalent to nt 48-98 in BCoV 3' UTR), a hypervariable region showing 60% nt sequence variation among group 2 coronaviruses [76], is nonessential for MHV replication although it plays a role in pathogenesis [68]. It is possible, however, that since the 2'-O-MT also binds sites downstream of the hypervariable region, i.e., within nt 1-55, as foot printing indicated, this interaction might be sufficient for the 2'-O-MT to perform a 3' end function in the deleted genome described by Goebel et al.

Assuming 3' end binding by the 2'-O-MT serves a function beneficial to the virus, as to what that function might be we envision several possibilities. First, 2'-O-MT may help initiate assembly of the replication-transcription complex by interacting with other viral replicase

proteins such as nsp 8-12 as suggested by Zust et al [68], or possibly viral structural proteins, or cellular proteins including mitochondrial aconitase [47], one or more of three mitochondrial heat shock proteins (HSP 40, HSP 60, HSP 70) [48], or hnRNP A1 [46], as shown by Leibowitz et al, or PABP as shown by Spangnolo and Hogue [21]. Such a complex might be required for initiation of negative-strand synthesis. Such a role is supported by an extensive genetic study with MHV which has shown the 3' terminal 45 nt probably interact with nonstructural proteins and the large loop of the 3' proximal BSL-PK to form an initiation complex for negative-strand synthesis [40, 68]. Second, 2'-O-MT may bind the 3' terminus and methylate structures at the genomic 5' end by virtue of genome cyclization. Alternatively, other structures within or at the 3' end of the genome may become methylated by 2'-O-MT. This idea is compatible with the suggestion that the 2'-O-MT could have evolved in CoV to protect viral RNA species from degradation by the virus-encoded nucleases NendoU (nsp 15) and ExoN (nsp 14). It has been shown that both enzymes, implicated in RNA processing, do not cleave methylated RNA species. Third, from the precedent that vaccinia virus 2'-O-MT VP39 binds the 3' end of ssRNA and serves as a co-factor in poly (A) synthesis suggests that this possibility may also function for the coronavirus 2'-O-MT.

Despite the fact that 2'-O-MT is shown to bind the viral 3' UTR, computer analysis of the amino acid sequence has not revealed any known RNA-binding domains for BCoV 2'-O-MT. The identity of RNA binding domains in many methyltransferases is unknown, but they are expected to vary widely in character since as a group they bind a wide variety of substrate targets [59]. Thus further studies are needed to determine the motif(s) on 2'-O-MT that binds CoV 3' UTR structures.

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CHAPTER III. CoV 2'-O-MT ACTIVITY IS REQUIRED FOR VIRUS REPLICATION.

INTRODUCTION

CoVs are positive-sense, single-stranded RNA viruses with a 5' terminal methylated cap and 3' terminal poly (A) tail. The presence of the 5' terminal cap structure on the viral genomic RNA and sgRNAs was first identified by T1-oligonucleotide fingerprinting for IBV and MHV in the early 80's [9]. However the enzymes involved in the capping process and the capping pathway along with the precise details of the cap structure itself have not been determined. Because of the presence of the cap structure on all CoV RNAs it has been presumed that CoVs use a cap-dependent translation mechanism.

The cap at the 5' end of most eukaryotic mRNAs functions to enhance the efficiency of initiation of translation as well as mRNA stability. The 5' cap serves as the binding site for initiation factor eIF4E which along with subunits eIF4A and eIF4G forms the initiation complex eIF4F and directs the binding of the 40S ribosome subunit to mRNA [52]. Moreover interactions between the cap bound eIF4G and poly (A) tail-bound poly (A)-binding protein (PABP) has been shown to induce 5'-3' RNA circularization that is believed to increase translation efficiency of RNAs [77]. The cap in eukaryotes is usually synthesized in the nucleus by three enzymes, a triphosphatase, a guanylyltransferase and a guanine-N7-methyltransferase (N7MT) [78]. Therefore, it has been presumed that capped RNA viruses replicating in the cytoplasm must provide their own enzymes for the capping reaction. Virus encoded methyltransferases have been shown to be involved in the third step of the capping process. In this step, viral N7MT adds a methyl group to the 7-nitrogen of guanine forming the cap-0 structure. Many viral RNAs have a cap-1 structure which requires an additional methylation event on the ribose hydroxyl of the first transcribed nucleotide. This function is performed by a second enzyme, the 2'-O-MT.

Just recently the capping machinery and pathways in CoVs have been partially identified. Nsp13 was postulated to be involved in the capping reaction and to function both as a 5'-triphosphatase and a helicase [56, 57]. Nsp14 has been just recently identified as a guanine-N7-methyltransferase [58]. The guanylyltransferase involved in the CoV capping reaction has not yet been identified. Nsp16 has been predicted by bioinformatic analysis to be a 2'-O-MT [35, 50]. It has been presumed that the CoV 2'-O-MT is responsible for methylation of the 5' cap structure of viral RNAs similarly to the homologous enzyme NS5 in the flavivirus family [51]. A putative catalytic tetrad K-D-K-E present in the Dengue virus 2'-O-MT (NS5) and related 2'-O-MT of vaccinia virus (VP39) [63] is also present in the BCoV 2'-O-MT. In the BCoV 2'-O-MT catalytic amino acids are K46-D130-K170-E203. Methyltransferase activity for methylation of the cap-0 structure was shown to be associated with nsp16 of the feline coronavirus [65]. While it has been presumed that capping of the CoV genome and sgRNAs is necessary for cap-dependent translation of viral RNAs, and as a corollary, that the viral 2'-O-MT enzyme activity is necessary for virus replication these have not yet been experimentally demonstrated. To determine if 2'-O-MT is involved in enhancing viral RNA translation we used the rabbit reticulocyte lysate (RRL) and the wheat germ extract (WGE) systems to translate Drep1 RNA (DI RNA) which expresses a fusion protein as a test molecule. The fusion protein is comprised of the N-terminal 95 aa of nsp1 and the entire N protein. To assess the function of 2'-O-MT, the 2'-O-MT was synthesized *in-vitro*, purified and added back to a translation reaction with Drep1 RNA and the function of 2'-O-MT was determined by analysis of fusion protein expression. Here we report that (i) 2'-O-MT enhances translation of DI-RNA *in-vitro* in a cap-dependent manner. (ii) 2'-O-MT activity is required for the 2'-O-MT function in translation enhancement of

DI RNA. (iii) 2'-O-MT activity is required for MHV replication *in-vivo* as determined by a mutagenesis study with the MHV reverse genetics system [79]. Alanine scanning mutagenesis of the 2'-O-MT conserved catalytic tetrad demonstrated the requirement for enzyme activity for virus replication. Mutations: K46A, D130A, K170A, E203A, whether single or multiple, were shown to block RNA synthesis and virus production.

MATERIALS AND METHODS

Constructs.

The construction of pDrep1 has been described previously [19]. T7 RNA polymerase transcripts of pDrep1 were used as templates for *in-vitro* translation assays (Figure 13.A). pDrep1 plasmid was also used to generate constructs for protein expression in rabbit reticulocyte lysate (RRL). The 2'-O-MT ORF was cloned into the Hpa1 and Mfe1 restriction sites of pDrep1 to generate construct pDrep1-MTwt (Figure 13.B). A 3xGlycine linker (3xGly) followed by 6xHistidine tag (6xHis) were placed at the C-terminus of 2'-O-MT ORF to enable purification of the *in-vitro* expressed protein on Ni-NTA columns. A 2'-O-MT mutant construct with 3 active site residues replaced by alanine (D130A, K170A and E203A) was generated by PCR overlap mutagenesis and named pDrep1-MTDKE (Figure 13.C). Both constructs contained the 5' proximal 155 nt of the BCoV genome at the 5' end and the 16 nt 3' UTR that represents the last 16 nt of the BCoV genome, and a 65 nt poly (A) tail at the 3' end. The 2'-O-MT ORF start codon was placed just downstream of the Hpa1 site. The 2'-O-MT stop codon was placed just upstream of the Mfe1 restriction site.

pD-RLuc construct (Figure 13.D) was used to generate a DI RNA transcript harboring the

Renilla luciferase (RLuc) ORF (K.M.Gustin, PhD Dissertation UTK 2008). RLuc protein was used as a control protein in the translation reactions. Oligonucleotides used for construction of plasmids are listed in Table 2.

RNA synthesis.

2 µg of Mlu I-linearized DNA plasmid was used for RNA synthesis. Uncapped RNAs (Drep1-MTwt, Drep1-MTDKE, D-RLuc and pppG-Drep1) and capped unmethylated RNA (GpppG-Drep1) were generated using T7 RNA polymerase (Promega) following the manufacturer's protocols. N7 methylated capped RNA (^{7m}GpppG-Drep1) was synthesized using the T7 Message Machine (Ambion). 2 U of RNase-free DNase (Promega) was added to the reaction mixtures to remove DNA template. RNAs were purified with the RNaid RNA purification kit (MP Biomedicals). RNAs were quantitated by spectrophotometry and their integrity evaluated by formaldehyde gel electrophoresis.

In vitro translation and purification of 2'-O-MTwt, 2'-O-MTDKE and RLuc.

In-vitro translation reactions of Drep1-MTwt, Drep1-MTDKE, and D-RLuc RNAs were performed using RRL (Promega). RNAs were translated individually in a 50 µl reaction volume following the manufacturer's protocol (Promega) with 500 ng of RNA template. Reactions were incubated at 30 °C and samples were collected at 90 min time point. Micrococcal nuclease (160 U/ml, Pharmacia) was added to the 50 µl sample with micrococcal nuclease buffer (50 mM Tris HCl (pH 8.8), 5 mM CaCl₂ and 0.1 mg/ml BSA) and reactions were incubated at 37 °C for 30 min to remove RNA followed by inactivation of micrococcal nuclease with 5 mM EGTA at 37 °C for 10 min. Micrococcal nuclease treated samples were flash frozen or subjected to Ni-NTA purification of the 6xHis-tagged proteins. For this the sample was diluted 1:10 with binding

buffer (1xPBS, 1x proteinase inhibitor cocktail (Roche), 10 mM imidazole) and 20 ul of pre-calibrated Ni-NTA beads were added and incubation continued for 30 min followed by three washes with 1000 ul of binding buffer. Bound 6xHis-tagged proteins were eluted with binding buffer containing 300 mM imidazole. The eluted protein was either flash frozen or used directly in translation reactions to determine its effects (Figure 13.E).

In certain experiments translation of Drep1-MTwt, Drep1-MTDKE, and D-RLuc RNAs were performed using wheat germ extract (WGE) (Promega) following the manufacturer's protocol with 500 ng RNA template in 50 ul reaction volumes. The protein samples were further purified following the protocol for proteins synthesized in the RRL system.

RRL-based assay for 2'-O-MT function in Drep1 RNA translation regulation.

10% of 2'-O-MTwt sample either from Ni-NTA elution or directly from translation mix was added to the translation reaction with Drep1 RNA as a template. Translations of RNA templates ^{7m}GpppG-capped Drep1, GpppG-capped Drep1 or uncapped pppG-Drep1 in a 50 µl reaction were performed following the manufacturer's protocol (Promega). 500 ng of RNA was added to reaction mix containing 35 µl of RRL, 40 U of RNasin (Promega), 1 µl of 1mM amino acid mixtures (-) Met, 2 µl of ³⁵S-Met (20 µCi), purified protein or protein translation mix (10% or as indicated on the figure) and S-adenosyl-L-Methionine (SAM) (100 uM or as indicated on figure). Reactions were incubated at 30 °C and 5 µl aliquots were taken at the indicated times, frozen on dry ice and stored at -80 °C (Figure 13.E).

The WGE-based assay was performed following the RRL-based assay as described above, except 10% of WGE translated proteins elute or protein translation mix was used in translation reaction as the protein source. Reactions were carried out at 25 °C in 50 ul reaction

volume containing 130 mM potassium acetate (KAc) and 5 μ l aliquots were collected at the indicated times.

SDS loading dye was added to 5 μ l aliquot samples, heated at 100 °C for 3 minutes and loaded onto a 12% polyacrylamide stacking gel for SDS-PAGE. Following electrophoresis, gels were fixed in 30% methanol-10% glacial acetic acid for 1 hour, impregnated with 128 Enhance Fluor (Perkin Elmer) for 1 hour, precipitated in water for 30 minutes and dried using an Air-gel dryer (BioRad). Results were analyzed with autoradiography and quantitated using a Phosphoimager.

Construction of 2'-O-MT mutants used for MHV reverse genetics.

The primers used to generate 2'-O-MT mutants are listed in Table 2. The F plasmid of MHV-A59 containing 2'-O-MT ORF (nt 20846 to 21742) was used to introduce mutations into the 2'-O-MT active site with PCR overlap mutagenesis. PCR products were cloned between the AflII and SphI restriction enzyme sites, which are naturally present within the F segment. The single active site mutants K46A (nt 20981 to 20983), D130A (nt 21233 to 21235), E203A (nt 21452 to 21454) were generated and one mutant with all four active site residues (KDKE) changed to alanine was made (Figure 17.A). All plasmids were routinely sequenced to verify the presence of mutations.

Assembly of a full-length MHV-A59 infectious clone (icMHV-A59).

The reverse genetics system for MHV-A59 which was developed by the R. Baric laboratory was a kind gift from R. Baric. The system was used as described [79]. Briefly, the plasmids containing each of the 7 MHV fragments (MA, MB, MC, MD, ME, MFwt, MG) and 4 plasmids with F segment mutations (MFK46A or MFD130A or MFE203A or MFKDKE) (Figure

17.A) were electroporated into Lucigen cells (E.cloni 10G classic) and grown to a high concentration at 30 °C. Purified DNA was digested or double digested following the established protocol and DNA digestion products were gel purified and ligated with T4 DNA ligase at 4 °C overnight. The ligation reaction products were *in-vitro* transcribed with the mMessage Machine (Ambion). N gene DNA was amplified from the MHV G clone with primer pairs flanking the N gene and the upstream primer containing a T7 promoter (forward primer T7-MHVA59NATG, reverse primer MHVA59Npol (A) (Table 2) and transcribed *in-vitro* with the mMessage Machine (Ambion).

RNA transfection.

The full-length RNA transcripts of the MHVwt and mutant constructs (MHVK46A, MHVD130A, MHVE203A and MHVKDKE) were electroporated three times (850 V at 25 µF) into 800 µl of BHK-MHVR cells at a concentration of 10^7 cells/ml. The transfected BHK-MHVR cells were seeded with DBT cells in a 75-cm² flask and incubated at 37 °C for up to 72 hr. Virus progeny were then passaged or blind-passaged in DBT cells 3 times at 48 hr intervals. Cells were observed for MHV cytopathology and cell fusion for up to 72 hr post-transfection for all MHVwt construct and mutants MHVK46A, MHVD130A, MHVE203A, and MHVKDKE.

RESULTS

2'-O-MT enhances SAM-dependent translation of ^{7m}GpppG-capped Drep1 RNA in a concentration-dependent manner.

To determine if 2'-O-MTwt activity contributes to viral RNA translation, translation enhancement was monitored in RRL, a method commonly used to examine the influence of

RNA-binding proteins on translation [80]. We chose Drep1 RNA as a template for the *in-vitro* assay. 2'-O-MTwt expressed *in-vitro* in the RRL system was His-tag purified and added to subsequent translation reactions with Drep1 RNA templates variously capped and with SAM (100 uM, or as indicated on the figure), the methyl group donor for 2'-O-MT. The function of 2'-O-MTwt was determined by measuring the amount of fusion protein product (Figure 13.E). Compared to RLuc as a control, 2'-O-MTwt enhanced the translation of Drep1 RNA with the 5' terminal methylated cap (^{7m}GpppG) but not with the 5' terminal un-methylated cap (GpppG) or with uncapped (pppG) RNA (14.A and B) indicating that the observed enhancement of ^{7m}GpppG-capped RNA translation was a function of the 2'-O-MTwt.

To determine if the enhancement of ^{7m}GpppG-capped RNA translation was dependent on the concentration of 2'-O-MTwt, the amount of translation was measured against a titrated 2'-O-MT. Because the amounts of 2'-O-MT expressed and purified from RRL system were small (in the range of 50-200 ng/ 50 ul reaction (<http://www.promega.com/paguide/chap5.htm>), the exact concentration of protein was not determined. The reaction was set up following the standard conditions as described above, except that the mixture of tested protein and control protein was used to maintain the same added volume of protein source but modify the tested protein volume in each sample. The final amount of protein preparations used for each 50 ul ^{7m}GpppG-capped Drep1 RNA translation reaction was in order: 100% RLuc and 0% MTwt, 66% RLuc and 33% MTwt, 33% RLuc and 66% MTwt. The results shown in Figure 14.C and D indicate that the 2'-O-MT dependent ^{7m}GpppG-capped Drep1 RNA translation enhancement increases with increased 2'-O-MT concentration in the reaction mix.

CoV encoded 2'-O-MT is predicted to be SAM-dependent. The potential SAM binding

motifs Motif I (the glycine-rich region between $\beta 1$ and αA that interacts with the amino-acid part of SAM), and II (an acidic loop between $\beta 2$ and αB that interacts with ribose hydroxyls) were predicted in the BCoV 2'-O-MT (Figure 16). To determine whether the translational enhancement depends on SAM concentration, translation of variously capped Drep1 RNAs was tested in the presence of increasing concentrations of SAM. The used SAM concentrations ranged from 0 to 200 μM . The results indicate that 2'-O-MT translation enhancement was SAM concentration dependent (Figure 14.E and F). This enhancement was observed only with ^7mGpppG -capped Drep1 RNA and not with $GpppG$ -capped or uncapped RNA.

A mutated 2'-O-MT fails to enhance translation of ^7mGpppG -capped RNA *in-vitro*.

To determine whether the amino acids in the putative catalytic tetrad play a role in the enhancement of translation observed with 2'-O-MT, mutations in the protein active site were made and tested. For this wild type 2'-O-MT was substituted with its mutant 2'-O-MTDKE (expressed form Drep1-MTDKE) (Figure 13.C) in which 3 of the 4 putative active site residues were mutated to alanine (D130A, K170A, E203A). No enhancement of translation of DI RNA was observed (Figure 15.A and B). The data indicate that 2'-O-MT putative active site is required for the enhancement of translation of ^7mGpppG -capped Drep1 RNA *in-vitro*.

To determine the generality of the cap requirement for optimal BCoV 2'-O-MT-enhanced translation and to exclude the possibility that RRL is lacking translation regulation factors required for efficient cap-dependent translation, translation of ^7mGpppG -capped Drep1 RNA was tested in the WGE to which was added 2'-O-MTwt or the 2'-O-MTDKE mutant. Consistent with the RRL results, 2'-O-MTwt enhanced translation of ^7mGpppG -capped Drep1 RNA whereas 2'-O-MTDKE did not (Figure 15.C and D). Thus, the results from both *in-vitro* systems

demonstrate that the BCoV 2'-O-MT^{wt} functions as an enzyme to enhance the translation of ^{7m}GpppG-capped Drep1 replicon RNA.

Viral 2'-O-MT activity is required for CoV replication.

To examine the biological effects of viral 2'-O-MT enzyme function on MHV replication, the full length MHV-A59 virus genome was assembled by reverse genetics to contain a single mutation or multiple mutations within the KDKE catalytic tetrad and tested for virus replication. For this, genomes containing the single mutations K46A, D130A or E203A, and one genome containing all 4 active site residues K46-D130-K170-E203 mutated to alanine were made and tested (Figure 17.A). None of the mutants electroporated into BHK-MHVR cells seeded with DBT cells induced the development of CPE within 72 hr post electroporation. No CPE developed for each mutant through a third cell passage or through a third supernatant passage onto fresh cells. Assembled virus with wild-type 2'-O-MT sequence (encoded in fragment F) however yielded wild-type virus within 24 hr of electroporation (Figure 17.B). These results suggest that the 2'-O-MT putative active site is necessary for virus replication and that each of the three (of the four) active site residues tested are required for productive virus infection.

DISCUSSION

Previous work [65] has demonstrated that at least one function of nsp16 is to methylate a 5' cap-0 structure on coronaviral genomic RNA. To test whether this 2'-O-methylation does indeed (i) function to enhance translation of viral RNA, and (ii) is required for CoV replication, we did two sets of experiments. In the first, the 2'-O-MT function in translation was tested *in-*

vitro in RRL and WGE systems. In the second, mutations were made by reverse genetics in the 2'-O-MT active site in the MHV genome and the reconstituted virus was tested for viability.

In the first set of experiments we chose transcripts of Drep1 as the template for the *in-vitro* translation assays since it in many essential ways mimics the viral genome. *In-vitro* translated 2'-O-MTwt was purified and added back into *in-vitro* translation reactions with variously capped Drep1 RNAs. These were RNA templates with (i) the 5' terminal methylated cap ^{7m}GpppG-RNA, a type 0 cap, (ii) the 5' terminal un-methylated cap GpppG-RNA, and (iii) the uncapped pppG-RNA. The function of 2'-O-MT was determined by measuring the amount of fusion protein expressed from the RNA template. The assays demonstrated that the BCoV encoded 2'-O-MT enhanced the translation of Drep1 transcripts. The function of 2'-O-MT methyltransferase in translation enhancement has been demonstrated previously for *c-mos* mRNA in oocytes [81]. Our results are consistent with the report of Decroly et al [65] that the CoV 2'-O-MT has the requirement of a guanine-N7 methylated cap structure (cap-0) for 2'-O-metylation to a cap 1 structure. The enhancement that we observed, furthermore, is 2'-O-MT concentration dependent and SAM concentration dependent. Since the enhancement of translation with ^{7m}GpppG-capped RNA correlates with an increase in the concentration of exogenously added SAM, there is a possibility that in addition to serving as a methyl group donor, SAM by binding to the 2'-O-MT may induce conformational changes that result in a higher affinity and/or specificity of 2'-O-MT for its target RNA. This kind of effect of SAM on methyltransferase function has been identified for T4 Dam methyltransferase [82].

The enhancement of translation was further demonstrated to be a function of the 2'-O-MT active site since a mutant of 2'-O-MT with the DKE sites converted to alanine did not

enhance translation of ^{7m}GpppG-capped RNA in either RRL or WGE systems. These results together demonstrate the CoV 2'-O-MT functions to enhance translation of the genomic DI RNA and by analogy the virus genome, which lends assurance that translation of the virus genome, the first step in genome replication is accomplished.

The 2'-O-MT activity was also shown by reverse genetics analyses to be required for MHV replication. Mutations of the individual 2'-O-MT active site residues K46A, D130A and E203A, or changes of all four catalytic tetrad residues to alanine were shown to block MHV replication. The data indicate that active viral 2'-O-MT is necessary for viral RNA synthesis and productive viral infection. It is possible that in addition to its effects on translation the viral 2'-O-MT mutations could have more directly affected steps in the viral replication cycle that occur after transcription. For example capping of newly synthesized positive-strand RNAs could have been directly affected leading to a global decrease in the number of viral subgenomic mRNAs to be translated. In addition, other potential functions of the 2'-O-MT in viral RNA synthesis need to be considered as well. The 2'-O-MT may be a multifunctional protein. It may methylate other sites in the viral or even cellular RNAs as yet to be discovered. It also may function in RNA proofreading, recombination as proposed by Snijder et al [35], or due to its binding to the 3' end of the genome, in minus-strand synthesis or in replicase complex assembly (Chapter II and Appendix E). Several eukaryotic viruses, such as poxviruses, alphaviruses, flaviviruses and reoviruses have been shown to encode cap 2'-O-MT activities. However the exact role of the cap 2'-O-methylation in the virus life cycle of these viruses is still unknown, even though this function was shown to be required for their replication. Mutations that abolished the N7 and 2'-O-MT activities of WNV [83, 84] and N7 methyltransferase activity of Sindbis virus [85] had a

detrimental effect on viral replication. An important role for 2'-O-MT in Dengue Virus replication was shown for NS5 protein in which mutations in both the methyltransferase active site and SAM binding abolished viral replication [86]. However, because the more likely predominant importance of N7MT over 2'-O-MT function in translation as shown for other viruses [87], the necessity of active 2'-O-MT in MHV replication may suggest the additional functions for that protein.

Finally, the central function of cap-dependent translation in CoVs is now being re-examined in the light of the fact that there may be an alternative internal ribosome entry mechanism for translation of CoV genomic and sgRNAs (unpublished data). Our current interpretation is that there are two functional mechanisms for initiation of translation, cap-dependent, and cap-independent internal ribosomal entry. In light of these new data, more studies are needed to establish the importance of CoV cap-dependent translation.

**CHAPTER IV. OVER-EXPRESSION OF BCoV 2'-O-MT INHIBITS DI RNA
REPLICATION IN CIS BUT HAS NO DRAMATIC EFFECT ON VIRUS
REPLICATION, TRANSCRIPTION OR TRANSLATION IN TRANS.**

INTRODUCTION

Successful viral infection requires specific interactions between the genomic RNAs and viral proteins. The timing and amount of protein expressed from the viral genome involved in RNA synthesis are typically tightly regulated. Lack of regulation may result in inhibition of virus replication by disturbing the proper balance of required viral components. Moreover a lack of regulation may result in overproduction of viral toxic proteins or peptides that may in turn kill the host cell. In RNA viruses a number of mechanisms have evolved that regulate differential expression of viral proteins. These include, but are not limited to, polyprotein processing, ribosomal frame shifting, regulated sgRNAs synthesis, internal-ribosome entry site (IRES), ribosomal read-through to translate viral proteins encoded in 3' proximal ORFs of multicistronic RNAs, re-initiation of translation, leaky scanning, and ribosomal shunting [52]. In CoV, several of these mechanisms have been identified. These include a -1 ribosomal frameshift within ORF1 [33], regulated template switching for sgRNAs synthesis [88], an internal ribosomal entry for translation [89, 90] and subcellular compartmentalization.

The CoV encoded 2'-O-MT has been recently shown to methylate the cap structure localized at the 5' terminus of the viral genome and sgRNAs [65]. This function seems to contribute to translation regulation (Chapter III). However, the unique characteristic of BCoV 2'-O-MT binding to genomic 3' proximal structures and the necessity for 2'-O-MT in virus replication could indicate that the 2'-O-MT is multifunctional and is directly involved in viral genome replication in ways other than cap-O methylation. Other functions have been proposed for CoV 2'-O-MT mediated regulation which include (i) 2'-O-MT directed methylation of selected internally localized nucleosides in virus and/or cellular RNAs as part of the complex of

unique CoV RNA-processing enzymes [35] and (ii) a direct involvement of 2'-O-MT in RNA synthesis based on the analysis of the MHV temperature sensitive mutants [67].

To explore if and how higher than normal levels of *in-vivo* expressed 2'-O-MT will affect BCoV virus replication and to study the possible additional functions of 2'-O-MT in virus replication, a system was designed and tested that would potentially over-express 2'-O-MT from a DI RNA sgRNA template. With the use of this system the observation was made that 2'-O-MT was over-expressed and that this over-expression correlated with *in-cis* repression of 2'-O-MT ORF-containing DI RNA accumulation. The decrease in DI RNA accumulation strictly depended upon 2'-O-MT activity since over-expressed mutants of 2'-O-MT did not affect DI RNA accumulation similarly. Curiously, 2'-O-MT over-expression had no effect on the accumulation of viral sgRNAs or on their translation. How this 2'-O-MT activity relates to other functions, such as enhancement of translation of viral messages has not been determined. Additionally, *in-vivo* over-expressed 2'-O-MT was also shown in preliminary experiments to be associated with the viral proteins N protein and nspl by His-tag pull-down experiments (Appendix E). We propose that 2'-O-MT functions as part of the replicase complex assembled at the 3' terminus of DI RNA.

MATERIALS AND METHODS

Plasmid constructs.

A cDNA cloned 2.2kb BCoV defective interfering RNA (DI RNA) with a 30 nt reporter named pDrep1 has been previously described (Figure 13.A) [23]. pDrep1 was further modified to carry the promoter (an RdRp template switching signal) for subgenomic mRNA synthesis and

was named pDrepIS12.7gD [73]. To test this DI RNA mini-replicon as a vector for over-expression, a 923 cloned fragment containing the upstream 18 nt intergenic sequence (IS) for N mRNA expression (AATAATCTAAACTTTAAGG) and the 2'-O-MT cistron (BCoV genome nt 20,595-21,494) with C-terminal 3×Glycine linker (3xGly) and 6×Histidine Tag (6xHis), flanked by BamHI (nt 1667) and KpnI (nt 1865) restriction enzyme sites, was cloned into the corresponding enzyme sites in the N protein coding region that replaced the 198 nt fragment of pDrep12.7 construct. The construct was named pDrep12.7-MTwt (18.A). Additional constructs were made that express 2'-O-MT mutants. These were pDrep12.7-MTK46A (in which the 2'-O-MT carried a single mutation of the enzyme active site at amino acid 46, K46A) and pDrep12.7-MTDKE (in which the 2'-O-MT carried mutations of three predicted active site residues D130A, K170A, E203A) (Figure 18.A). The mutations were introduced into 2'-O-MT ORF by PCR overlap mutagenesis. Oligonucleotides used in this study are listed in Table 3.

RNA synthesis.

For synthesis of uncapped transcripts of Drep12.7, Drep12.7-MTwt, Drep12.7-MTK46A and Drep-12.7-MTDKE, 2 µg of Mlu I-linearized plasmid DNA were transcribed in a 100 µl reaction mix containing 40 U of T7 RNA polymerase (Promega) using the manufacturer protocol. RNA was purified with the RNaid RNA purification kit (MP Biochemicals) and quantified spectrophotometrically and RNA integrity was evaluated by formaldehyde gel electrophoresis.

RNA extraction and Northern blotting to measure DI RNA replication and viral genomic and subgenomic mRNA synthesis.

Northern blot analysis for quantitating DI and viral RNAs was performed as previously described [91, 92]. Briefly, HRT cells at ~80% confluence in a 35-mm dish (8×10^6 cells per dish) were infected with BCoV at a multiplicity of 10 PFU per cell and transfected with 500 ng of DI RNA transcript 1 h later using Lipofectin (Invitrogen). For passage of progeny virus, supernatant was harvested at 48 hpi and 500 μ l was used to infect fresh cells a step called virus passage (VP1). Supernatant from VP1 at 48 hpi was used to infect new cells (VP2) (Figure 18.B). RNA was extracted with Trizol (Invitrogen) at the indicated times post transfection and stored in water at -80°C . One fourth of the RNA sample was used per lane for formaldehyde gel electrophoresis. ~ 1 ng of RNA transcript was used as a marker. RNA was transferred to Hybond N+ nylon membrane (Amersham) by vacuum blotting and membranes were UV irradiated for 2 min (UV stratalinker 1800). Northern blots were probed with γ - ^{32}P -end labeled oligonucleotide probe at 5×10^5 cpm/ pmol. The HSVgD (+) probe (Table 3) was used for detecting DI RNA and its subgenomic RNA transcripts and the N (+) probe (Table 3) was used for detecting DI RNA and viral genomic and sgmRNAs. Probed blots were analyzed by autoradiography and quantified with a Phosphor Imager (Molecular Dynamics) or scanned films were analyzed with ImageJ (<http://rsb.info.nih.gov/ij/>).

Detection of the over-expressed 2'-O-MT in cells.

To measure the level of 2'-O-MT over-expression, total 2'-O-MT expressed following transfection was compared to that expressed during infection alone. For this, cell lysates were prepared from (i) uninfected, untransfected cells, (ii) cells infected with BCoV only and (iii)

BCoV-infected cells that had been transfected with Drep12.7-MTwt RNA. At the indicated times post-transfection cells were washed with ice-cold 1xPBS and lysed for 20 min on ice with 500 μ l / 8×10^6 cells of modified RIPA buffer (50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1x proteinase inhibitor complete (Roche), 1 mM EDTA, 10 mM imidazole). The lysate was clarified by centrifugation for 10 min at 14,000 rpm at 4 °C. Lysates were concentrated by ammonium sulfate precipitation (90% saturation). The material obtained from 3×10^5 to 2.3×10^6 cells/ lane was subjected to SDS-PAGE in gels of 12% polyacrylamide and analyzed with Western Assay with 2'-O-MT specific polyclonal antibodies.

Purification of the over-expressed 2'-O-MT.

For purification of over-expressed 2'-O-MT, DI RNA was transfected into BCoV helper virus-infected HRT cells in 25cm² flasks as described above. At the indicated times post-transfection, cells were lysed and the lysate clarified. 6xHisTag 2'-O-MT was bound to pre-calibrated Ni-NTA resin (Pierce) (100 μ l suspension/1500 μ l cell lysate) for 30 min at 4 °C followed by three washes with RIPA buffer (1 ml each). Bound 6xHis-tagged 2'-O-MT was eluted with RIPA buffer containing 300 mM imidazole (100 μ l).

Western analysis of the over-expressed 2'-O-MT and concurrent expression of viral proteins in virus-infected-transfected cells.

Western analysis of experimental samples was done following the Pierce protocol (<http://www.piercenet.com/Proteomics/browse.cfm?fldID=8259A7B6-7DA6-41CF-9D55-A6C14F31193>). Briefly, experimental samples were subjected to 12% SDS-PAGE followed by transfer to a nitrocellulose membrane (Hybond-ECL, Amersham). The membrane was blocked with 1:5 dilution of Super Block Blocking Buffer (Pierce) and then exposed to a rabbit 2'-O-MT

specific polyclonal antibody (1: 2000; Proteintech Group Inc.). After incubation with goat anti-rabbit IgG (H+L) antibody coupled with horseradish peroxidase (1: 30000 dilution) the signal was detected with Super Signal West Pico Substrate (Pierce). The analysis of Western data was made with the ImageJ program (<http://rsb.info.nih.gov/ij/>).

Similarly, Western analysis of N protein was done to measure N protein expressed from sgRNA7. For this, samples were probed with N protein specific polyclonal antibodies (Proteintech Group Inc.) to analyze the effect of 2'-O-MT over-expression on translation of sgRNAs.

RESULTS

2'-O-MT over-expression from DI RNA has no significant effect on virus genome replication or transcription.

The over-expression of 2'-O-MT from the BCoV DI RNA was tested (i) to determine whether over-expression of this regulatory protein has any effect on virus replication *in-trans* or on viral RNA translation, (ii) to determine if the over-expression of 2'-O-MT has any *in-cis* effect on DI RNA replication or translation, and (iii) to determine if over-expression of 2'-O-MT from a DI RNA can be used to establish a simple method for purifying 2'-O-MT from mammalian cells for further study (described in Chapter II and Appendices D and E). This over-expression system mimics natural virus infection and thus it may be expected that protein expressed from DI RNA will localize at the virus replication compartment and be available to the viral replication machinery *in-trans*. To determine the expression of 2'-O-MT from DI RNA a Western Assay using BCoV 2'-O-MT specific antibodies was done on

cellular extracts from (i) uninfected HRT cells, (ii) BCoV-infected HRT cells and (iii) BCoV-infected and Drep12.7-MTwt-transfected cells (Figure 19). Based on ImageJ analyses there was ~60 fold increase in 2'-O-MT accumulation in cells infected with BCoV and transfected with 2'-O-MT expressing DI RNA at 24hr post transfection when compared with infected cells only. To determine whether 2'-O-MT over-expression has an effect on virus replication, a Northern blot analysis was done initially to compare the abundance of viral RNA species among the three sets of cells (Figure 20.A). Analysis of the Northern gel shows no obvious differences in viral sgRNAs abundance suggesting that 2'-O-MT over-expression had no detrimental effect on virus replication and transcription. Thus, from these analyses, it appears that 2'-O-MT can be expressed from a sgRNA derived from DI RNA (Figure 20.A) and that this expression does not cause an overall detrimental effect on virus replication. Moreover, the 60-fold over-expression as observed by Western analysis suggests that this system would be useful for obtaining quantitative amounts of protein product in mammalian cells for further biochemical analysis.

2'-O-MT over-expression inhibits DI RNA replication in-cis.

To determine whether 2'-O-MT over-expression has an effect on DI RNA accumulation in-cis a Northern analysis of DI RNA accumulation was made. BCoV-infected HRT cells were transfected with transcripts of Drep12.7-MTwt, expressing wild type 2'-O-MT or with transcripts of Drep12.7-MTK46A or Drep12.7-MTDKE, expressing 2'-O-MT mutants, and evaluated by Northern analysis with a probe that recognizes the reporter sequence HSVgD which is localized downstream of 2'-O-MT ORF. Drep12.7 vector was used as a control for no expression of 2'-O-MT. Total RNAs were extracted at 1, 24 and 48hr post-transfection, and at viral passage 1 (VP1) and viral passage 2 (VP2). The Northern analysis shown in Figure 21.A and B revealed that

over-expression of the wild type 2'-O-MT reduced DI RNA replication by roughly 40-60% at VP1 when compared to transcripts of Drep12.7 which carries no 2'-O-MT gene, and reduced DI RNA replication by roughly ~20% of the wild type at VP2. However, over-expression of the 2'-O-MT mutants 2'-O-MT_{DKE} (Figure 21.A) and 2'-O-MT_{K46A} (Figure 21.B) led to DI RNA abundance similar to levels of Drep12.7 transcript alone, indicating no inhibition of DI RNA replication. These results indicate that inhibition of DI RNA replication is due only to the enzymatic activity of 2'-O-MT and not other modifications in the construct.

Over-expressed 6xHis-tagged 2'-O-MT can be purified from infected cells.

To determine whether over-expressed 6xHis-tagged 2'-O-MT can be purified from infected cells, a first step was to determine the expression profile at different times post transfection (Figure 21.C). 2'-O-MT accumulated in cells up to ~48hr post transfection then decreased at later times (VP1, VP2) to ~40% of that observed at 48hr post transfection as measured by Western analysis with 2'-O-MT specific antibodies. A decrease in 2'-O-MT levels observed at the later times likely reflects the decrease in sgRNA synthesis arising from impaired mini-replicon replication.

Western Assay analysis of the effects of over-expressed 2'-O-MT on the translation of viral RNAs *in-vivo*.

To evaluate if over-expressed 2'-O-MT regulates *in-trans* translation of viral sgRNAs the N protein (nucleocapsid) expression from sgRNA7 was tested by Western assay with N protein specific antibodies (Figure 20.B). The results showed no difference in N protein accumulation in cells when comparing infected cells that were transfected with Drep12.7 RNA, Drep12.7-MT_{wt} RNA or Drep12.7-MT_{DKE} RNA and analyzed at 20 min, 40 min, 60 min, 180

min, 360 min and 540 min post-transfection. The results indicate that 2'-O-MT over-expression does not affect the translation of sgRNA7, at least at these times and under the tested conditions. It is still possible that 2'-O-MT may be involved in the translation regulation of viral RNAs but the effects may be too small to be detected by this method. Unfortunately, we are not able to examine if 2'-O-MT over-expression regulates neither translation of genomic RNA nor its function *in-cis* on DI RNA translation from which it is being expressed.

DISCUSSION

In addition to its function in the methylation of the 5' terminal cap structure, other functions have been proposed for CoV 2'-O-MT. These include the following: (i) A 2'-O-MT methylation of selected internally located nucleotides in viral or cellular RNAs as the part of the complex of unique CoV RNA processing enzymes [35]. The idea agrees with a reported inhibition of RNA cleavage by nsp15 of RNAs containing 2'-O-MT methylated bases [39]. (ii) A direct involvement of 2'-O-MT in RNA synthesis was suggested based on the analysis of the MHV temperature sensitive mutants [67]. (iii) The unique binding of the BCoV 2'-O-MT to the 3' terminus of the viral genome as described in Chapter I suggests that the BCoV 2'-O-MT could have functions independent of its role in cap methylation and translation enhancement as presented in Chapter III. It may, for example, be directly involved in viral genome replication by playing a role in initiating negative strand synthesis. The evidence for methyltransferase functions in RNA synthesis has been observed in other viruses. Sindbis Virus nsp2 methyltransferase-like domain, so far not considered to be a functional MT, has been implicated in regulation of minus-strand synthesis by providing a scaffold for interactions with RNA and

other nonstructural proteins and stabilization of replicase in the promoter region for minus-strand synthesis [91]. Moreover Sindbis Virus encoded nsp1 which contains a functional methyltransferase that methylates 5' terminal cap structure in viral messages was also suggested to regulate minus-strand synthesis by interacting with nsp4 [92]. A role for the methyltransferase-like domain of alfalfa mosaic virus (AMV) in the synthesis of viral negative-strand RNA was also proposed [93]. (iv) From the precedent that vaccinia virus 2'-O-MT VP39 binds the 3' end of mRNA and serves as a co-factor in poly (A) synthesis [94, 95] it is possible that the CoV 2'-O-MT may also have similar function in plus-strand RNA synthesis.

Because of these postulated functions for the CoV 2'-O-MT we anticipated that over-expression of the 2'-O-MT from the BCoV-dependent DI RNA replicon would likely cause detrimental effects on some aspect(s) of virus replication. Surprisingly, disruption of helper virus replication and translation was not found, and the apparent inhibition of the parental DI RNA was not sufficient to prevent over-expression. The expression of His-tagged 2'-O-MT from DI RNA was ~60fold of that from the virus infection alone. At this time, the inhibition of the DI RNA *in-cis* is not fully understood. The inhibition does not occur when the 2'-O-MT with the active site mutation (whether single amino acid substitution or three active site residues of total four were mutated) is over-expressed in the system. This indicated that the observed result of the down regulation of DI RNA replication is due only to the enzymatic activity of 2'-O-MT.

Since the DI RNA is both a template for translation and for replication and since *in-cis* translation of N and partial nsp1 have been shown previously to be required for DI RNA replication a higher rate of translation could be inhibitory for replication since both can not occur simultaneously on the same molecule. Alternatively, an overabundance of protein products due

to 2'-O-MT enhancement of translation of viral RNAs (genome, sgRNAs or DI RNA molecule per se) could secondarily inhibit DI RNA accumulation. That is, enhanced translation could result in over production of viral proteins or peptides that would in turn impair efficient DI RNA replication.

Alternatively, since both the initiation of the negative-strand synthesis and viral RNA polyadenylation require an interaction of viral polymerase with the 3' UTR, which contains the 45-nt promoter, secondary or tertiary structure modifications of that region may regulate recognition by the viral polymerase and consequently regulate transcription efficiency. BCoV 2'-O-MT binding to the viral 3' terminal sequence as shown in Chapter I along with the accompanying RNA structure modifications suggest that 2'-O-MT may function in replication complex assembly and regulate viral RNA synthesis. The 2'-O-MT interactions with viral RdRp identified in *in-vitro* studies seems to support our above hypothesis (Figure 22). More extensive studies are required to determine the exact function of the BCoV 2'-O-MT in DI RNA and genome replication.

CHAPTER V. FUTURE DIRECTIONS

INTRODUCTION

CoV are important pathogens of mammals and birds. However until the outbreak of SARS in 2003 there was little interest in the wider medical community for identification of antivirals against this family of viral pathogens. The rapid spread of SARS-CoV to over 30 countries in just a few months with an accompanying high mortality rate (10% world wide) defined a serious new public health problem since no therapies or vaccines were available (http://www.who.int/csr/sars/country/table2004_04_21/en/index.html). This new infectious disease stimulated wide-ranging interest in the molecular biology of CoVs. That includes virus binding and entry, replicase function and *cis*-acting replication and packaging signals, since all can reveal potential novel anti-coronavirus targets. Several CoV-encoded proteins have been suggested as potential targets for antiviral therapy. These included but are not limited to the spike protein used for viral attachment [96], nsp3 and nsp5 which are viral proteases that process products from ORF1 [97], and the RdRp [98]. In order to better understand the virus replication processes and identify effective antiviral targets, an extensive study of CoV biology is needed. This dissertation addresses some of the requirements for BCoV replication. The focus of this research is the 2'-O-MT protein, nsp16. Since our study has shown the 2'-O-MT to be essential for CoV replication, it is becomes a potential target for antiviral therapy. 2'-O-MT inhibitors may be potent antiviral agents. Additionally, the unique binding of 2'-O-MT to 3' terminal genomic structures may reveal an unusual function of 2'-O-MT in that region of genome in virus replication. Thus the 3' terminal *cis*-acting 8mer-BSL may be an important RNA target for antiviral development. More detailed analyses are necessary to better understand CoV replication that will in turn allow us to develop better prevention and treatment methods.

Identification of the 2'-O-MT RNA binding motif.

The BCoV 2'-O-MT has been shown experimentally to be an RNA binding protein. Despite this fact, computer analysis of the amino acid sequence has not revealed any known RNA-binding domains. The identity of RNA binding domains in many methyltransferases is unknown but they are expected to vary widely in character since they bind a wide variety of substrate targets [59]. Studies are needed to identify the motif(s) on 2'-O-MT that are involved in binding the cap structure and viral 3' terminal structures. Because of the importance of 2'-O-MT function in virus replication the identified RNA binding motifs may, in addition to its active site blockers, identify potential targets for antiviral therapy.

Identification of potential 2'-O-MT function in polyadenylation.

I postulate that the unique 2'-O-MT binding site at the 3' region engages a substrate for protein function independent or in addition to the cap methylation and translation regulation. The 2'-O-MT enzymatic function in this region of the viral RNA, if identified, may indicate the direct involvement of 2'-O-MT in regulation of transcription and/or replication of the viral genome. VP39, the vaccinia virus-encoded 2'-O-MT, has been shown in addition to its cap methylation function to be a processivity factor for mRNA tail elongation by VP55 [94], a poly (A) polymerase. Based on analogy to VP39 and the unique binding characteristics of the BCoV 2'-O-MT to the 3' terminal genomic structures, I hypothesize that 2'-O-MT functions in polyadenylation of viral RNAs. Moreover, the interaction of the BCoV 2'-O-MT with nsp12 (viral RdRp) identified in my His-Tag pull-down experiments (Figure 22) would seem to further support this hypothesis since the interaction resembles those between vaccinia virus VP39 and

VP55 [95]. Identification of such a 2'-O-MT function could yield an explanation for the characteristic 2'-O-MT binding to the genomic 3' terminus.

Identification of cellular proteins that bind the bulged stem-loop/pseudoknot and the octamer-associated bulged stem-loop *cis*-acting elements in both the negative and positive-strands.

The gel shift analysis and UV-cross-linking analysis identified complexes of cellular proteins binding to the BCoV genomic 3' terminus in both the positive and negative strands. Two unidentified cellular proteins with molecular weights of ~40kDa and ~100kDa were shown to bind the BSL-PK positive-strand (Figure 23.C), and three with molecular masses ~45kDa, ~70kD and ~100kDa were shown to bind the positive-strand 8mer-BSL (Figure 25.A). In the negative-strand RNA binding analysis three cellular proteins with molecular weights of ~45kDa, ~70kDa and ~85kDa were shown to interact with the BSL-PK (Figure 23.E) and three cellular proteins of ~25kDa, ~45kDa and ~70kDa were shown to interact with 8mer-BSL (Figure 25.D). These cellular proteins may play important roles in the functions of the BSL-PK and 8mer-BSL. Identification of these proteins will be important for determining the function of these elements in virus genome replication.

Identification of viral protein that binds the positive-strand of the octamer-associated bulged stem-loop.

UV-cross-linking experiments identified a viral protein of ~36-40kD in infected cell lysates that binds the positive-strand of the 8mer-BSL (Figure 25.A). Based on its molecular weight the possible CoV genome-encoded candidate might be 2'-O-MT (~34kD), nsp15 (~42kD) or the

matrix structural protein M (~38kD). The identity of the putative viral protein could be determined by Western analyses with protein specific antibodies, or by mass spectrometry of RNA pulled-down protein as described in Chapter II.

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APPENDICES

Appendix A

Preliminary identification of the viral and cellular proteins binding to the positive and negative-strands of the bulged stem-loop/pseudoknot.

INTRODUCTION

The BSL-PK structure has been determined by enzyme probing and confirmed to exist at the 5' terminus of 3' UTR of BCoV and its close relative MHV [30, 31]. The BSL-PK consists of a 5' proximal *cis*-acting BSL that maps just downstream of the N stop codon and a *cis*-acting PK structure that maps 63 nt downstream of the N stop codon. The BSL and the PK were shown to overlap in sequence suggesting that they can not exist simultaneously. Thus the two structures were proposed to form one functional element with alternative conformations functioning as a molecular switch (BSL-PK) [31]. The BSL-PK region has been shown to be required for DI RNA replication [30, 99]. The BSL-PK is conserved among group 1 and 2 CoVs both in higher order structure and location but not in the primary sequence. This element was postulated to function in the plus-strand based on the co-variation of bases to maintain G-U base pairs [30]. However, since *mfold* analysis also predicts a similar although less stable structure ($\Delta G = -34.70$) in the negative-strand (Figure 23.D), a BSL-PK function in the negative-strand can not be ruled out. Additionally, both structures are functionally equivalent within group 2 CoV since they can be switched between BCoV and MHV viruses without affecting DI RNA replication [96]. Until now only one cellular protein, hnRNP A1 has been identified as binding the BSL-PK [45]. No viral proteins were found to bind this region. The cellular proteins interacting with viral RNA

structures probably play an important role in virus replication and may have regulatory functions in translation and RNA synthesis. This makes their identification important. To begin to explore the interactions of BSL-PK with cellular and viral proteins, gel shift and UV-cross-linking assays were performed using the BCoV-infected and mock-infected cell lysates as the protein source and positive and negative-strands of the BSL-PK as the probes. Cellular proteins with molecular weights of ~40kDa and ~100kDa were found to bind the positive-strand BSL-PK and three with molecular weights of ~45kDa, ~70kDa and ~85kDa were found to bind the negative-strand BSL-PK. Further analyses are needed to identify the five proteins. No viral proteins were found to bind either the positive or negative-strand of BSL-PK under the conditions used.

MATERIALS AND METHODS

Constructs and synthesis of RNA probes.

Cloning and synthesis of the positive-strand BSL-PK probe were described in Chapter I. For generation of the negative-strand BSL-PK RNA probe the same construct was used but it was linearized with EcoRI restriction enzyme and transcribed with SP6 RNA polymerase following the manufacturer's protocol. The *mfold* predicted secondary structures of the positive and negative-strands of the BSL-PK are shown in Figure 23.A and D, respectively.

Gel shift assay.

Gel shift assays were performed as described in Chapter I except that cell lysates were used instead of *E.coli*-expressed protein.

Cell lysates.

Cell lysates were prepared as described by Thompson et al [97]. Briefly, cells in the 35 mm dish were infected with BCoV at a MOI of 10 viruses per cell or mock-infected. At 6 hpi cells were washed with cold 1x PBS and collected by scraping and centrifugation. Cell pellets were resuspended in four cell volumes of lysis buffer (10 mM HEPES [pH 7.5], 3 mM MgCl₂, 14 mM KCl, 5% glycerol, 0.5%-1% NP40, 1 mM DTT, 1x Complete Protease Inhibitor [Roche]) and lysed on ice for 20 min followed by Douncer homogenization. Nuclei were removed by centrifugation at 3000 rpm for 10 min at 4 °C and aliquots were flash frozen and stored at -80 °C. Protein concentration was determined by the Bradford assay.

UV-cross-linking.

UV-cross-linking was performed as described by Pestova et al [102]. Briefly, α -³²P-labeled RNA probe at $\sim 2 \times 10^5$ cpm was incubated in a 20 μ l reaction volume with the BCoV-infected or mock-infected cell lysate (~ 15 μ g protein total) for 10 min at 37 °C in binding buffer as described for EMSA in Chapter I. The sample was further UV irradiated on ice at 3 mwatts/cm² for 45 min using a UV Stratalinker 1800. Subsequently the sample was RNase treated with 2.5 U RNase A, 100 U RNase T1 and 0.2 U RNase CV1 (Ambion). Samples were subjected to SDS-PAGE and gels dried on Gel-Air Dryer (BioRad) were analyzed by autoradiography.

RESULTS AND DISCUSSION

To identify the cellular and viral proteins that bind BSL-PK in both positive-strand and negative-strand the gel shift assays were performed using the BCoV infected and mock infected cell lysates. Gel shift analyses identified two cellular complexes that bind to the positive-strand

BSL-PK (Figure 23.B). No viral proteins were identified under tested conditions. To determine the specificity of the protein binding to BSL-PK competition of binding was tested with cold probes that included (i) specific competitor (an unlabeled BSL-PK RNA) and (ii) a presumed non-specific competitor (tRNA). The analyses showed that the unlabeled BSL-PK probe competes with radio-labeled BSL-PK probe for protein binding. tRNA does not compete with the radio-labeled BSL-PK probe suggesting specificity and affinity of that interaction (Figure 23.B). To determine the number and approximate molecular weights of the proteins in these two complexes the UV-cross-linking analysis was performed. UV-cross-linking identified two proteins with molecular masses $\sim 40\text{kDa}$ and $\sim 100\text{kDa}$. Further analyses are required to identify the bound proteins (Figure 23.C).

Binding of proteins to the negative-strand BSL-PK was similarly investigated by UV-cross-linking. UV-cross-linking analysis identified three cellular proteins with molecular masses of $\sim 45\text{kDa}$, $\sim 70\text{kDa}$ and $\sim 85\text{kDa}$ (Figure 23.E). The identities of these proteins are currently unknown. It is possible from size and binding characteristics that the 45kDa protein is the previously identified PTB. Further analyses such as Western blot with PTB specific antibodies and mass spectrometry will be required to verify this hypothesis.

No viral proteins were found to bind either the positive-strand or negative-strand of BSL-PK under the test conditions used.

Appendix B

Preliminary identification of the viral and cellular proteins binding to the positive and negative-strands of the the octamer-associated bulged stem-loop.

INTRODUCTION

The predicted structure of the 3' terminal 162 nt of genome that contains the 8mer-BSL and a 45 nt long promoter for negative-strand synthesis has been shown by enzymatic probing for BCoV (Chapter I) and its close relative MHV [32]. Mutagenesis analysis of MHV DI RNA suggested that the 8mer-BSL is important for DI RNA replication. However, analysis of the function of 3' terminal sequences by MHV reverse genetics demonstrated that a large fraction of the 8mer-BSL is not required for replication since deletion of nt 46-156 has little effect on replication. Although it is dispensable for replication it does contribute strongly to pathogenesis in the animal [76]. The very 3' terminal 45 nt of MHV genome was shown to be sufficient for minus-strand synthesis [68]. Until now only cellular proteins have been identified that bind the positive-strand and negative-strand the 3' UTR 3' terminal structures of group 2 CoV. These include the mitochondrial aconitase [47], three mitochondrial heat shock proteins (HSP 40, HSP 60, HSP 70) [47], hnRNP A1 [45], PTB and PABP [21].

To explore the interactions of the BCoV 8mer-BSL with cellular and viral proteins, gel shift and UV-cross-linking assays were carried out with BCoV-infected or mock-infected cell lysates. Both the positive and negative-strand 162 nt probes of the 8mer-BSL were used. Cellular proteins with molecular weights of ~45kDa, ~70kD and ~100kDa and one viral protein of

~40kDa bound the positive-strand 8mer-BSL. Cellular proteins of ~ 25kDa, ~45kDa, ~70kDa and ~85kDa bound the negative-strand 8mer-BSL. No viral proteins were found to bind the negative-strand of the 8mer-BSL.

MATERIALS AND METHODS\

Constructs and synthesis of RNA probes.

Cloning of the 162 nt octamer-associated bulged stem-loop (8mer-BSL) probe (nt -1 to -162), the 61 nt 8mer-BSL-left probe (nt -101 to -162) and the 101 nt 8mer-BSL-right probe (nt -1 to -101) was described in Chapter II. RNA synthesis for positive-strand probes was described in Chapter II. The negative-strand RNA probe was transcribed from the same pGEM construct but with the use of EcoRI-linearized plasmid DNA and SP6 RNA polymerase. The predicted secondary structures of the tested RNAs are shown in Figure 24, 25.A and D.

Cell Lysates, Gel Shift assay, UV-cross-linking assay.

The methods used were described in Appendix A.

RESULTS AND DISCUSSION

To identify cellular and viral proteins binding the 3' UTR elements of the BCoV genome, gel shift assays were employed. The 3' terminal 162 nt of genome that contains 8mer-BSL region and a 45 nt long promoter for negative-strand synthesis were tested for binding with BCoV-infected and mock-infected cell lysates. The results showed formation of the only one protein complex almost identical in both the uninfected and infected cell lysates (Figure 24). However, UV-cross-linking experiments identified a viral protein of ~36-40kD in the infected

cell lysate that interacts with the positive strand of the 8mer-BSL (Figure 25.A) with both left and right halves of 8mer-BSL probe (Figure 25.A through C). The identity of the putative viral protein is unknown at this time. Based on molecular masses, possible candidates are the 2'-O-MT (~34kD), nsp15 (~42kD), and the matrix protein (~38kD). Additionally, three unidentified cellular proteins of ~45kDa, 70kD and 100kDa bound the 8mer-BSL in the positive strand (Figure 25.A through C). All three were also shown to interact with the 8mer-BSL-left probe (Figure 25.B) and two (~45kDa and ~100kDa) with the 8mer-BSL-right probe (Figure 25.C).

Since it is possible that the 8mer-BSL functions in both the positive and negative-strands, binding of cellular and viral proteins to the negative-strand 8mer-BSL was also tested. In the UV-cross-linking analysis cellular proteins of ~ 25kDa, ~45kDa, ~70kDa and ~85kDa but no viral proteins were found to bind (Figure 25.D). Further analyses will be required to identify the bound proteins.

Appendix C

Preliminary identification of the binding of nsp1, nsp14 and N protein to the positive and negative-strands of the 3'UTR.

Since we were unable to detect the binding of viral proteins to regions in the 3' UTR other than the 8mer-BSL structure, in either the positive and negative-strand, we chose to use an alternative approach for identification of these proteins. For this viral proteins expressed and purified from E.coli were used in our assays. We used gel shift experiments to identify viral proteins binding to the BCoV 3' UTR structures. The proteins used were: nsp1 (pET-28a(+)) vector), nsp14 (pGEX-4T vector) and N protein (pET-SUMO-6xHis vector). The results are presented on the Figure 26. The gel shift analysis revealed the interaction of BSL-PK in positive-strand with nsp14, but no interaction was detected with nsp1 or N protein (Figure 26.A). The negative-strand of BSL-PK interacted with nsp1 and nsp14 but not with N protein (Figure 26.B). For 8mer-BSL region the interaction of nsp1 (Figure 26.D) and nsp14 (Figure 26.C) with the positive-strand probe was identified. The negative-strand of the 8mer-BSL was shown to interact with nsp1 and nsp14 (Figure 26.E). The importance and function of these interactions in virus replication remains to be determined.

Appendix D

Phosphorylation status of *in-vitro* and *in-vivo* expressed 2'-O-MT.

INTRODUCTION

Protein phosphorylation in both prokaryotes and eukaryotes is a common post-translational modification that modulates protein function and hence controls a number of cellular processes. Different levels of phosphorylation induce changes in protein conformation that may affect protein binding and enzymatic activity. In many viruses protein phosphorylation has been shown to regulate transcription and replication, RNA binding, binding with other proteins, and virus assembly. In CoV, several phosphorylation sites have been identified on the N protein which is believed to play important roles in virus replication and virion assembly [103-105]. Phosphorylation sites have been mapped within methyltransferase domains of cellular and viral enzymes that have been shown to regulate enzymatic activity. Examples include the methyltransferase of yellow fever virus [98], the glycine N-methyltransferase [99], the murine DNA methyltransferase [100], NSUN2 [109], and EZH2 [110]. Eight potential phosphorylation sites are predicted by bioinformatics in BCoV 2'-O-MT (NetPhos 2.0), and two of them are conserved in all groups of CoV (Table 4).

Here we show that BCoV 2'-O-MT expressed in a mammalian host cell line is not detectably phosphorylated. Hence, its binding to RNA when purified from E.coli is probably not a phosphorylation-dependent artifact.

MATERIAL AND METHODS

In-vivo expression of BCoV 2'-O-MT and *in-vitro* translation of BCoV 2'-O-MT.

The protocol for *in vivo* expression of the BCoV 2'-O-MT has been described in detail in Chapter IV. *In-vitro* translation of the BCoV 2'-O-MT has been described in detail in Chapter III.

BCoV 2'-O-MT dephosphorylation with calf intestinal alkaline phosphatase (CIP) and Western analysis of protein electrophoretic mobility.

In-vivo expressed 2'-O-MT purified from $\sim 4 \times 10^6$ cells was treated with CIP following the manufacturer's protocol (NEB). As a control 20 μ l of RRL translated 2'-O-MT sample was subjected to CIP treatment. Briefly, the proteins were incubated with CIP buffer (50 mM Tris-HCl pH 8, 1 mM $MgCl_2$) for 10 min at 30 °C followed by addition of 30 U of CIP and incubation continued for an additional 15 min at 30 °C.

CIP untreated and treated 2'-O-MT preparations were subjected to 12% SDS-PAGE electrophoresis followed by transfer to a nitrocellulose membrane for Western blot analysis with 2'-O-MT specific polyclonal antibody as was described in Chapter IV. The protein phosphorylation status was determined by analysis of protein mobility on SDS-PAGE.

RESULTS AND DISCUSSION

Since the initial studies for RNA binding were done with *E.coli* expressed 2'-O-MT and since *E.coli* expressed proteins are not phosphorylated, it is not known whether the binding properties were artificial for lack of proper phosphorylation modifications. Hence, the analysis of phosphorylation status of BCoV 2'-O-MT synthesized in the mammalian host cells was

anticipated. To determine the phosphorylation status of BCoV 2'-O-MT expressed in mammalian cells and thus to determine if phosphorylation has anything to do with the binding of 2'-O-MT to the 3' terminal 162 nt region of the 289 nt BCoV 3' UTR 2'-O-MT was subjected to CIP treatment and subsequently analyzed. To express 2'-O-MT in mammalian host cells, HRT cells were infected with BCoV and His-tagged BCoV 2'-O-MT was over-expressed from DI RNA as described in Chapter IV, purified and subjected to CIP treatment. As a control, RRL translated 2'-O-MT was likewise subjected to CIP treatment. The CIP treated proteins were subjected to SDS-PAGE and immunoblotting was done with 2'-O-MT specific antibodies. CIP untreated samples were analyzed in parallel. No differences in migration between treated and untreated proteins were observed after CIP treatments indicating that neither the *in-vivo* expressed nor RRL expressed protein are phosphorylated (Figure 27). Due to the fact that electrophoretic mobility shifts are evident only for heavily phosphorylated proteins, there remains the possibility that 2'-O-MT is phosphorylated post-translationally at a single or only a few sites (Table 4). Thus, low level phosphorylation may not be observed here. Moreover, some modifications other than phosphorylation may be present. Therefore, further analyses are necessary, to establish modifications in 2'-O-MT in mammalian cells.

Appendix E

Preliminary identification of potential partnering proteins that co-purify with BCoV 2'-O-MT.

INTRODUCTION

The BCoV genome ORF1 is translated into two polyproteins pp1a of ~500kDa and pp1ab of ~700kDa, which undergo co- or post-translational proteolytic processing into sixteen nonstructural proteins (nsp 1-16). It has been postulated that the 16 protein products function together as the replicase-transcriptase protein complex for RNA synthesis. However, the composition of the replicase-transcriptase complex has not yet been established in detail. To determine if the 2'-O-MT functions as part of the replicase and to identify proteins that interact with it *in-vivo*, we employed a His-Tag pull-down assay. The *in-vivo* protein preparations were made using the DI RNA over-expression system described in Chapter IV. Analyses showed that the 2'-O-MT expressed *in-vivo* co-purifies with N protein and nsp1 (p28). The data suggest that 2'-O-MT makes up part of the viral replication-transcription complex.

By this assay we were not able to identify interactions between the 2'-O-MT and nsp2, nsp8, nsp9, nsp12, nsp13, nsp14, nsp15 or hnRNP proteins.

MATERIALS AND METHODS

Plasmids, RNA synthesis and over-expression of His-tagged 2'-O-MT *in-vivo*.

Plasmid construction, RNA synthesis and over-expression of His-tagged 2'-O-MT were described in detail in the Chapter IV.

Antibodies used in Western assay.

The BCoV 2'-O-MT, nsp1, nsp9, nsp14, nsp15 and N protein rabbit polyclonal antibodies were prepared by Proteintech Group Inc. (<http://www.ptglab.com/>). MHV nsp2, nsp8, nsp12, nsp13 rabbit polyclonal antibodies were a kind gift from M.R. Denison. hnRNP A/B (H-200): sc-15385 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc.

His-tag pull-down of 2'-O-MT and identification of co-purified viral proteins.

At 24 hr post transfection the over-expressed 6xHis-2'-O-MT was isolated from HRT cells and purified by affinity chromatography on Ni-NTA. The Ni-NTA purified 2'-O-MT was subjected to SDS-PAGE electrophoresis followed by Western analysis with antibodies to 2'-O-MT and nsp1, nsp2, nsp8, nsp9, nsp12, nsp13, nsp14, nsp15, N protein, hnRNP proteins (Figure 28.A). In certain experiments the protein preparation was treated with micrococcal nuclease prior to Ni-NTA binding to remove the any bound RNA templates. For this, the protein sample (cell lysate from 25cm² flask) was incubated with micrococcal nuclease (160 u/ ml) in micrococcal nuclease buffer (50 mM Tris-HCl, pH 8.8, 5 mM CaCl₂, 0.1mg/ ml BSA) for 30 min at 37 °C followed by micrococcal nuclease inactivation with 5 mM EGTA for 10 min at 37 °C. To determine the strength of the interactions between 6xHis-2'-O-MT and interacting proteins, 6xHis-2'-O-MT bound to Ni-NTA resin was subjected to 3 washes (1 ml each) with NaCl of 150 mM, 300 mM and 450 mM concentration.

RESULTS AND DISCUSSION

To determine if *in-vivo* expressed 2'-O-MT formed complexes with other viral proteins a His-tagged 2'-O-MT pull-down assay followed by immuno-identification of bound proteins was

used. HRT cells infected with BCoV and transfected with Drep12.7-MTwt RNA (Figure 28.A) were lysed at 24 hr post-transfection with modified RIPA buffer and purified on Ni-NTA resin. Non-specific interactions with the Ni-NTA resin were blocked by addition of 10 mM imidazole to RIPA buffer. The bound 2'-O-MT and its associated protein complexes were eluted with RIPA buffer containing 300 mM imidazole, concentrated by ammonium sulfate precipitation (90% saturation), and analyzed by SDS-PAGE. Purified 2'-O-MT preparations were analyzed by Western assays for the presence of co-purified proteins. Co-purified proteins were identified with polyclonal antibodies against individual proteins which included antibodies to nsp1, nsp2, nsp8, nsp9, nsp12, nsp13, nsp14, nsp15, N protein and hnRNP proteins (Figure 28.A).

Based on our analyses, N protein co-purifies with His-tagged 2'-O-MT expressed in infected cells (Figure 28.B). The interaction is observed only at physiological salt concentration ~150 mM NaCl (Figure 28.B, lane 4). At higher concentrations such as 300 mM and 450 mM NaCl (Figure 28.B, lanes 5 and 6) the interaction between 2'-O-MT and N is lost. Removal of RNA with micrococcal nuclease treatment prior to Ni-NTA binding did not eliminate the 2'-O-MT- N interaction (Figure 28.B, lane 3). This result suggests that the 2'-O-MT and N are held together by a protein-protein interaction, direct or indirect, and not through their binding to a common RNA molecule. To determine if the interaction between 2'-O-MT and N protein is a direct one, a His-tagged form of the N protein was expressed *in-vitro* (RRL) and incubated with *in-vitro* synthesized untagged 2'-O-MT. The co-purification experiments did not confirm an interaction between 2'-O-MT and N protein (data not shown). This result suggests that the observed *in-vivo* interaction is probably not direct and may involve an unidentified viral protein or cellular bridging protein.

Nsp1 was found to co-purify with 2'-O-MT (Figure 28.C, lane 2). The interaction was strong and was present after washes that contain up to 450 mM NaCl (Figure 28.C, lanes 4, 5 and 6). The interaction was also observed after micrococcal nuclease treatment (Figure 28.C, lane 3) which is indicative of a protein-protein interaction and not an interaction dependent on a commonly-bound RNA molecule. To determine if the interaction between 2'-O-MT and nsp1 protein is direct or indirect, a His-tagged form of nsp1 was expressed *in-vitro* (RRL) and incubated with *in-vitro* translated untagged 2'-O-MT. The co-purification experiments did not confirm an interaction between 2'-O-MT and nsp1 (data not shown). This result indicates a probably indirect protein-protein interaction between 2'-O-MT and nsp1.

One of the cellular proteins that have been shown to associate with CoV genomic RNA is hnRNP A1. hnRNP A1 is predominantly found in the nucleus but it was also found to shuttle to cytoplasm. In the nucleus hnRNP A1 functions in splicing and mRNA transport [101] while in the cytoplasm its function mostly contributes to translation regulation and mRNA turnover [112, 113]. In CoV hnRNP A1 has been proposed to function in genome circularization and transcription due to its binding characteristics [44][45]. However this theory has been not confirmed experimentally [102]. Alternatively hnRNP A1 could be involved in translation of viral RNAs. Since 2'-O-MT is involved in a translational control of viral RNAs by methylating a 5' terminal cap structure (Chapter III), we tested for potential 2'-O-MT interaction with hnRNP proteins. However, no interaction between 2'-O-MT and hnRNP proteins was identified *in-vivo* in purified 2'-O-MT protein complexes (data not shown).

Overall our data suggest that His-tagged 2'-O-MT purifies on Ni-NTA as part of a multicomponent complex that is more likely associated with transcription / replication of DI

RNA. Surprisingly we were not able to identify interactions between 2'-O-MT and other putative proteins that include nsp2, nsp8, nsp9, nsp12, nsp13, nsp14 and nsp15 (data not shown). The lack of detectable interactions however, may be due to assay limitations. The tested proteins are expressed in very low levels in infected cells especially these from ORF 1b and therefore they may be impossible to detect in pull-down assays if not over-expressed. Interestingly an interaction between 2'-O-MT and nsp12 (RdRp) although not detected *in-vivo* was identified *in-vitro* after translation of both proteins in RRL (Figure 22).

Appendix F

Tables and Figures.

Table 1. Taxonomy of coronaviruses.^a * unclassified coronaviruses. ^b[], year of isolation

Coronavirus		Disease	Notable features....
Group 1	Canine coronavirus	gastroenteritis	
	1A		
	Feline coronavirus	peritonitis, enteritis	FCoV causes chronic enteric infections
	Transmissible gastroenteritis virus	gastroenteritis	
	Chinese ferret badger coronavirus		DM95/2003
	Raccoon dog coronavirus GZ43/2003		
	1B		
	Human coronavirus 229E	nasopharyngitis	IRES for E protein translation
	Human coronavirus NL63	bronchiolitis and conjunctivitis	[2004], associated with Kawasaki disease
	Porcine epidemic diarrhea virus	gastroenteritis	
	Bat coronavirus (11 strains)		
	Bat coronavirus (33 strains)*		Australia and Germany isolates
	Rocky Mountain Bat Coronavirus (6 strains)*		
	Ferret enteric coronavirus MSU1*		
Group 2	2A		
	Human coronavirus OC43	nasopharyngitis	absent 2 ORFs for 4.9- and 4.8-kDa nsp
	Human coronavirus HKU1	chronic respiratory disease	[2005], described from an adult
	Murine hepatitis virus	hepatitis, encephalitis	acute and chronic demyelination
	Bovine coronavirus	enteritis	
	Porcine hemagglutinating encephalomyelitis virus	encephalomyelitis	
	2B		
	SARS coronavirus	pneumonia	newly emerged, lacks HE gene
	Bat SARS CoV (3 strains)*		probably source of the SARS
	Bat coronavirus HKU3*		
	2C		
	Bat coronavirus (BtCoV/133/2005)		
	Bat coronavirus HKU4-1		
	Bat coronavirus HKU5-1		
	2D		
	Bat coronavirus HKU9-1		
	ungrouped		
	Puffinosis coronavirus		birds from islands off SW coast of Wales
	Rat coronavirus	upper respiratory infections	
	Bovine coronavirus KV0516*		
	Bovine coronavirus KV0501*		
	Bat coronavirus*		R.aus/Australia/CoV000/2006
	Canine respiratory coronavirus*	respiratory disease	8.8-kDa nsp substitutes 4.9 and 4.8kDa nsp
	Equine coronavirus*	enteritis	
	Human coronavirus type 5*		
Group 3	Infectious bronchitis virus (31 strains)	pneumonia	Pheasant coronavirus included
	Turkey coronavirus (3 strains)	enteritis	
	Coronavirus SW1*		
	Munia coronavirus HKU13-3514*		
	Thrush coronavirus HKU12-600*		
	Bulbul coronavirus HKU11-796*		
Human coronavirus NO*			
Human enteric coronavirus strain 4408*			
Duck coronavirus*			[2005]
Parrot coronavirus AV71/99*			
Chicken enteric coronavirus*			
Goose coronavirus*			[2005], two extra ORFs downstream of N
Pigeon coronavirus*			[2005], one extra ORF downstream of N
Bat coronavirus (5 strains)*			
Bird droppings coronavirus*			
Guangxi coronaviridae*			

¹ Polarity with (+) defined as the same polarity as RNA. ² Sequence in bold; restriction enzyme sequence, intergenic sequence (IG); T7 promoter sequence. Underlined; mutation site. ³ Binding region in the BCoV genome. ⁴ Binding region in the MHV genome.

Oligonucleotide	Pol ¹	Sequence (5'-3') ²	Binding region in genome
pDrep1-BMT-5'	+	cgggg gttaacatg gctgcatctgactggaagcctggtat	20595-20621 ³
pDrep1-BMT/His-3'	-	gccc caattgttaatgatgatgatggtggccgccc gattacattaac catactgtcaccaac	21465-21491 ³
BMT-K46A-5'	+	gatgaatgttgctgcatatac	20717-20737 ³
BMT-K46A-3'	-	ttgacataactgagtatatgcagcaac	20717-20737 ³
BMT-D130A-5'	+	gatttgataatctctgcaatgta	20967-20989 ³
BMT-D130A-3'	-	tacattgcagagattatcaaatc	20967-20989 ³
BMT-K170A-5'	+	cagtgttgctatagcaataac	21089-21109 ³
BMT-K170A-3'	-	gttattgctatagcaaacctg	21089-21109 ³
BMT-E203A-5'	+	atgcttcttctagtgcagggt	21187-21207 ³
BMT-E203A-3'	-	accctgcactagaagaagcat	21187-21207 ³
MMT-Afl II- 5'	+	cggg cttaag ctctttagaatttgaaattgacgtg	19928-19960 ⁴
MMT-SphI 3'	-	gccc gcatgcat ggtttaccgtcaatttcggta	21493-21522 ⁴
MMT-5'	+	gctgctgctgactggaaacctgggt	20846-20870 ⁴
MMT-3'	-	tttgacatttactaggctatcgcca	21718-21742 ⁴
MMT-K46A-5'	+	gatgaatgttgctgcatatac	20968-20988 ⁴
MMT-K46A-3'	-	ttgacataattgagtatatgcagcaac	20975-21001 ⁴
MMT-D130A-5'	+	atctgataatttctgcaatgtacgacctct	21219-21249 ⁴
MMT-D130A-3'	-	ttagtaagagggtcgtacattgcagaaatt	21226-21255 ⁴
MMT-K170A-5'	+	gcagtgttgccatagcaataacagagtgttct	21339-21370 ⁴
MMT-K170A-3'	-	cgttccaagaaaactctgttattgctatggcaac	21344-21377 ⁴
MMT-E203A-5'	+	cgcctcttcaagtgcaggatttttgattggc	21439-21469 ⁴
MMT-E203A-3'	-	tttatgccaatcaaaaatcctgcacttgaagag	21442-21474 ⁴
T7MHVA59NATG	+	gtaatacgactcactatagatg tcttttgttctctgggc	29672-29687 ⁴
MHVA59Npol(A)	-	tttttttttttttttttttttttacacattagagtc	31019-31031 ⁴

Table 3. Oligonucleotides used in generation of constructs for the 2'-O-MT over-expression system.

¹ Polarity with (+) defined as the same polarity as RNA. ² Sequence in bold, restriction enzyme sequence; *italic*, T7 promoter; ***italic bold***, 6xHis-Tag sequence; underlined, mutation site. ³ Binding region in BCoV genome. ⁴ Binding region in pDrep12.7.

Oligonucleotide	Pol ¹	Sequence (5'-3') ²	Binding region
pD.12.7-MT-5'	+	cgggggatccaatatctaaactttaaggatggctgcatctgactggaagcctgg	20595-20617 ³
pD.12.7-MT/His-3'	-	cgggggtacctaataatgatgatgatgatgggtggccgccgccgattacattaaccatactgt	21472-21491 ³
BMT-K46A-5'	+	gatgaatgttgctgcatatac	20717-20737 ³
BMT-K46A-3'	-	ttgacataactgagtatatgcagcaac	20717-20737 ³
BMT-D130A-5'	+	gatttgataatctctgcaatgta	20967-20989 ³
BMT-D130A-3'	-	tacattgcagagattatcaaatc	20967-20989 ³
BMT-K170A-5'	+	cagtgttgctatagcaataac	21089-21109 ³
BMT-K170A-3'	-	gttattgctatagcaactg	21089-21109 ³
BMT-E203A-5'	+	atgcttctctagtcagggt	21187-21207 ³
BMT-E203A-3'	-	accctgcactagaagaagcat	21187-21207 ³
HSVgD(+)	-	gagagaggcatccgccaaggcatattg	1866-1893 ⁴
N(+)	-	ccagaacgatttccaaaggacgctct	29429-29454 ³
pDrep1-MT-5'	+	cgggggttaacatggctgcatctgactggaagcctggtat	20595-20621 ³
pDrep1-MT-3'	-	gcccgaattgtaataatgatgatgatgatgggtggccgccgccgattacattaaccatactgt	21472-21491 ³
RdRp-5'	+	cgggggttaacatgtcaaaagataactaatttttaaacgggttcgg	13318-13349 ³
RdRp/His-3'	-	cgggcaattgtaataatgatgatgatgatgggtggccgccgccctgcataactgcacttctaaa tacatgttc	16070-16098 ³

Table 4. NetPhos 2.0 server predicted phosphorylation sites in the BCoV 2'-O-MT (NC_003045).

14 phosphorylation sites in total were predicted in the BCoV 2'-O-MT (NC_003045). Two predicted phosphorylation sites, serine and tyrosine are conserved in all groups of CoVs. ¹CoVs used in analysis, identified by name, group number of 1, 2 or 3, and Pubmed accession number. ²Conserved serine context. ³Serine position. ⁴Serine score. ⁵Conserved tyrosine context. ⁶Tyrosine position. ⁷Tyrosine score (<http://www.cbs.dtu.dk/services/NetPhos/>).

Group/Name ¹	Serine Context ²	Position ³	Serine Score ⁴	Tyrosine Context ⁵	Position ⁶	Tyrosine Score ⁷
1 /TGEV-nc_002306	SVNTSSSEG	201	0.997	NKDLYELIQ	182	0.963
1 /HC-229E-nc_002645	SVNTSSSEA	200	0.996	NKKLYELVQ	181	0.643
1 /FCoV-nc_007025	SVNTSSSEG	200	0.997	NKDLYELIQ	181	0.963
1 /CCoV-ACJ63240	SVNTSSSEG	200	0.997	NKDLYELIQ	181	0.963
1 /PEDV-nc_003436	SVNTSSSEA	200	0.996	NKKLYELIQ	181	0.576
2 /BCoV-nc_003045	NANASSSEG	200	0.995	NAELYKLMG	181	0.910
2 /MHV-JMH-nc_006852	NVNASSSEG	200	0.996	NAELYSLMG	181	0.861
2 /HO-OC43-nc_005147	NANASSSEG	201	0.995	NAELYKLMG	182	0.910
2 /PHEV-nc_007732	NANASSSEG	200	0.995	NAELYKLMG	181	0.910
2 /Bat-CoV-nc_008315	NANASSSEG	200	0.995	SADLYKIMG	181	0.985
2 /Bat-KHU9-nc_009021	SVNSSSEA	200	0.996	SADLYAIMG	181	0.987
2 /SARS-nc_004718	NVNASSSEA	200	0.995	NADLYKLMG	181	0.937
3 /TurkeyCoV-nc_010800	AVNASSSEA	204	0.990	HENLYDIAQ	185	0.861
3 /IBV-nc_001451	AVNASSSEA	204	0.990	HEVLYDIAQ	185	0.765

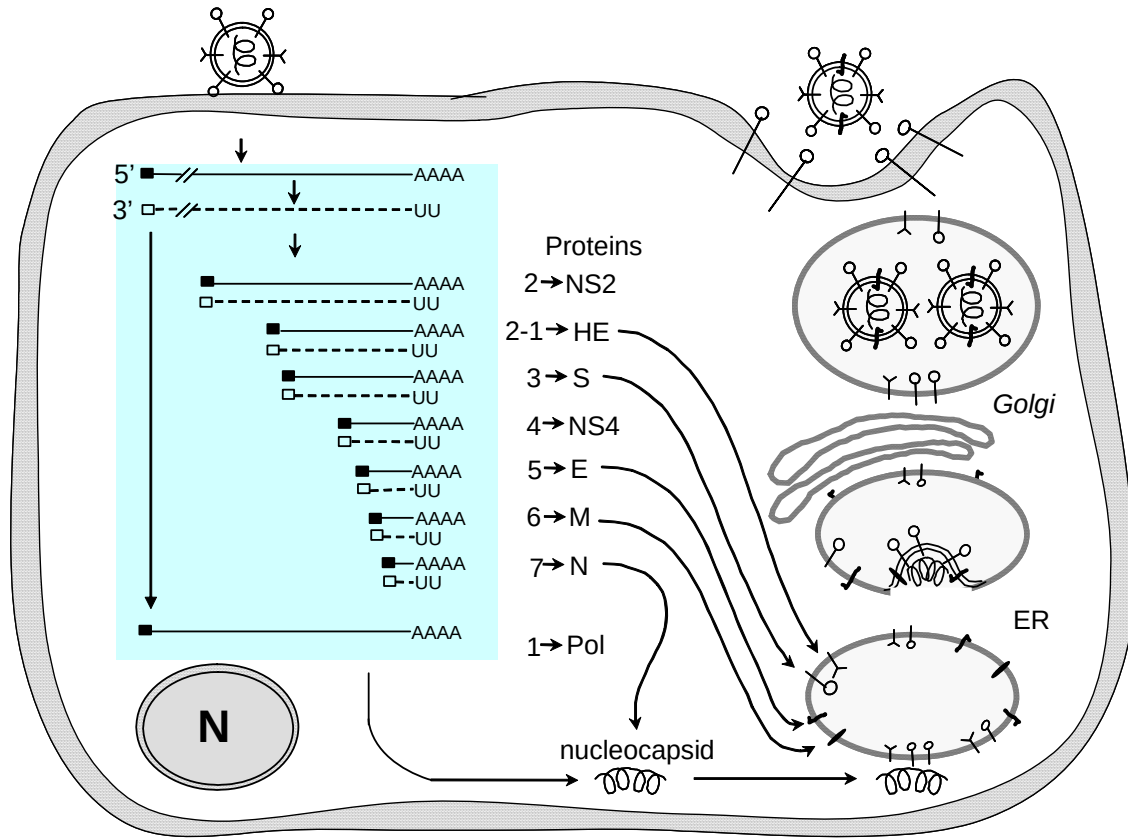


Figure 1. Coronavirus replication scheme.

Adapted from K. Holmes and M. Lai, *Fields Virology*, 1996; Sethna, Hung, Brian. 1989. PNAS 86:5626. CoV replication takes place entirely in the cytoplasm. Genome and sgRNA synthesis takes place in poorly identified double-membrane compartments (symbolized by the blue region). How the viral RNAs and proteins are transported in and out of the compartments is unknown. Viral proteins are synthesized on either free (N) or membrane-bound polyribosomes (M, E, S, HE and some of the replicase proteins). CoVs assemble by budding into the endoplasmic reticulum/ early Golgi membranes and virions exit via the exocytic pathway.

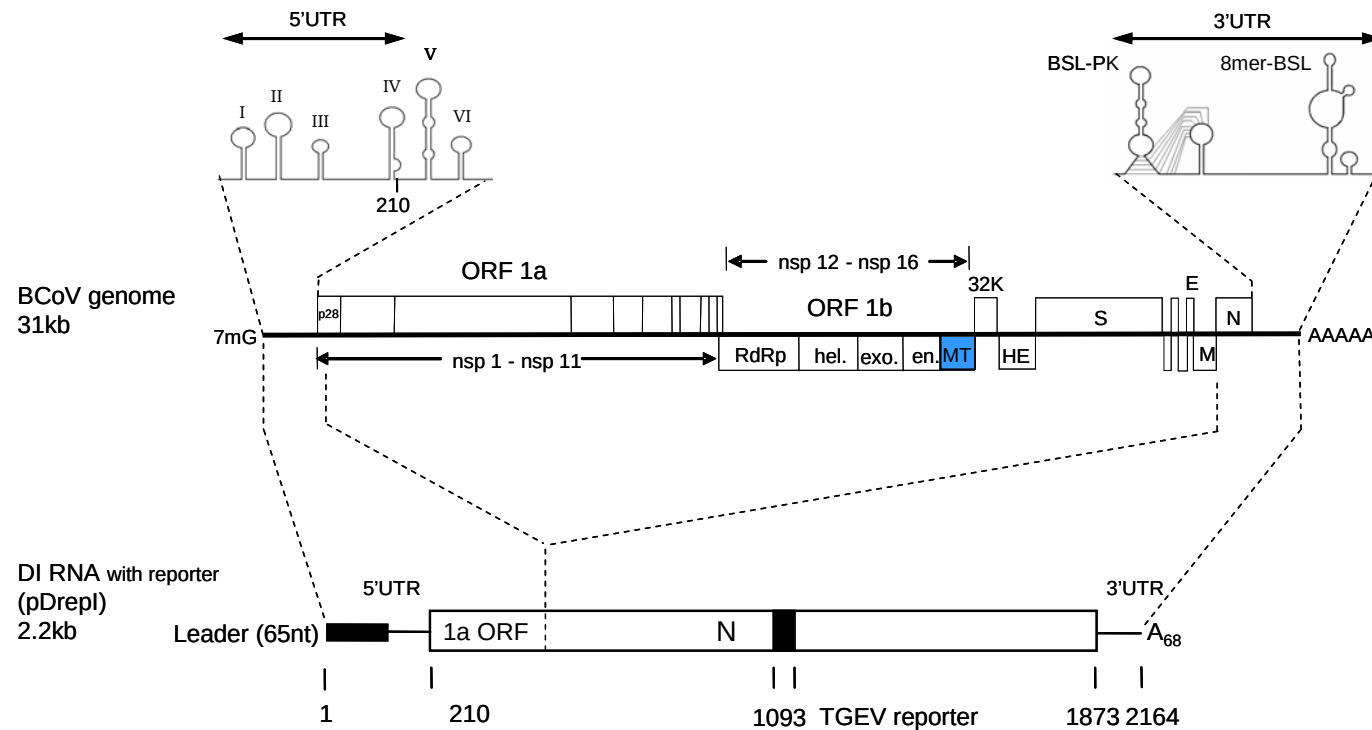


Figure 2. BCoV genome and DI RNA.

The genome is a positive-sense, single-stranded RNA with a 5' terminal cap structure and a 3' terminal poly (A) tail. The nonstructural proteins (nsps) are translated as a polyprotein from ORF1a/ORF1ab. The nsp16 cistron (the predicted gene for 2'-O-MT) is marked as blue box. Structural proteins including the spike glycoprotein (S), the envelope protein (E), the membrane protein (M), the nucleocapsid protein (N) and the hemagglutinin-esterase (HE) are encoded at the 3' terminus of the genome and expressed from sgRNAs. *cis*-acting secondary structures are found in both the 5' and 3' UTRs and in the nsp1 coding region (SLV-VI). The BCoV DI RNA consists of the 5' UTR, part of nsp1 from ORF1, the entire N gene and the 3' UTR. A TGEV in-frame reporter of 30 nt has been inserted into the N gene sequence to facilitate quantification of replication by Northern assay. This cloned, modified DI RNA construct is called pDrep1.

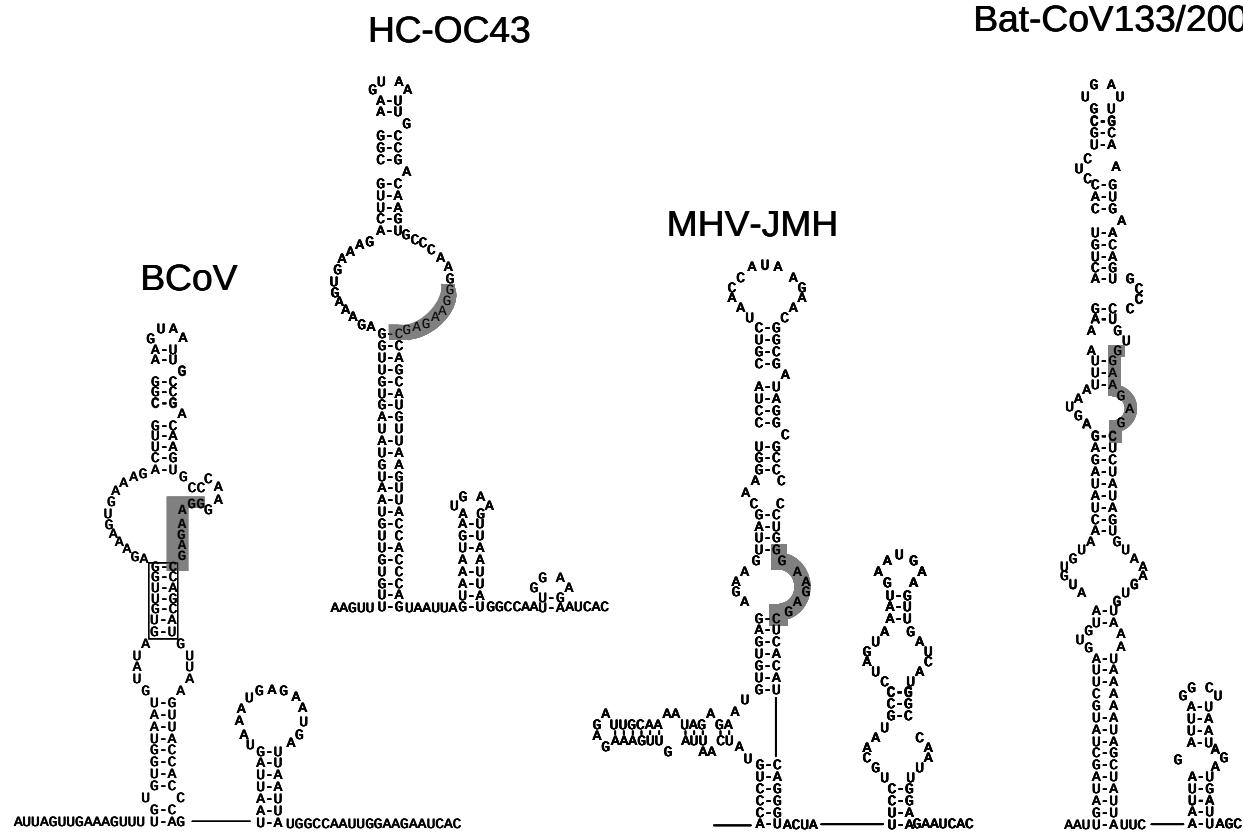
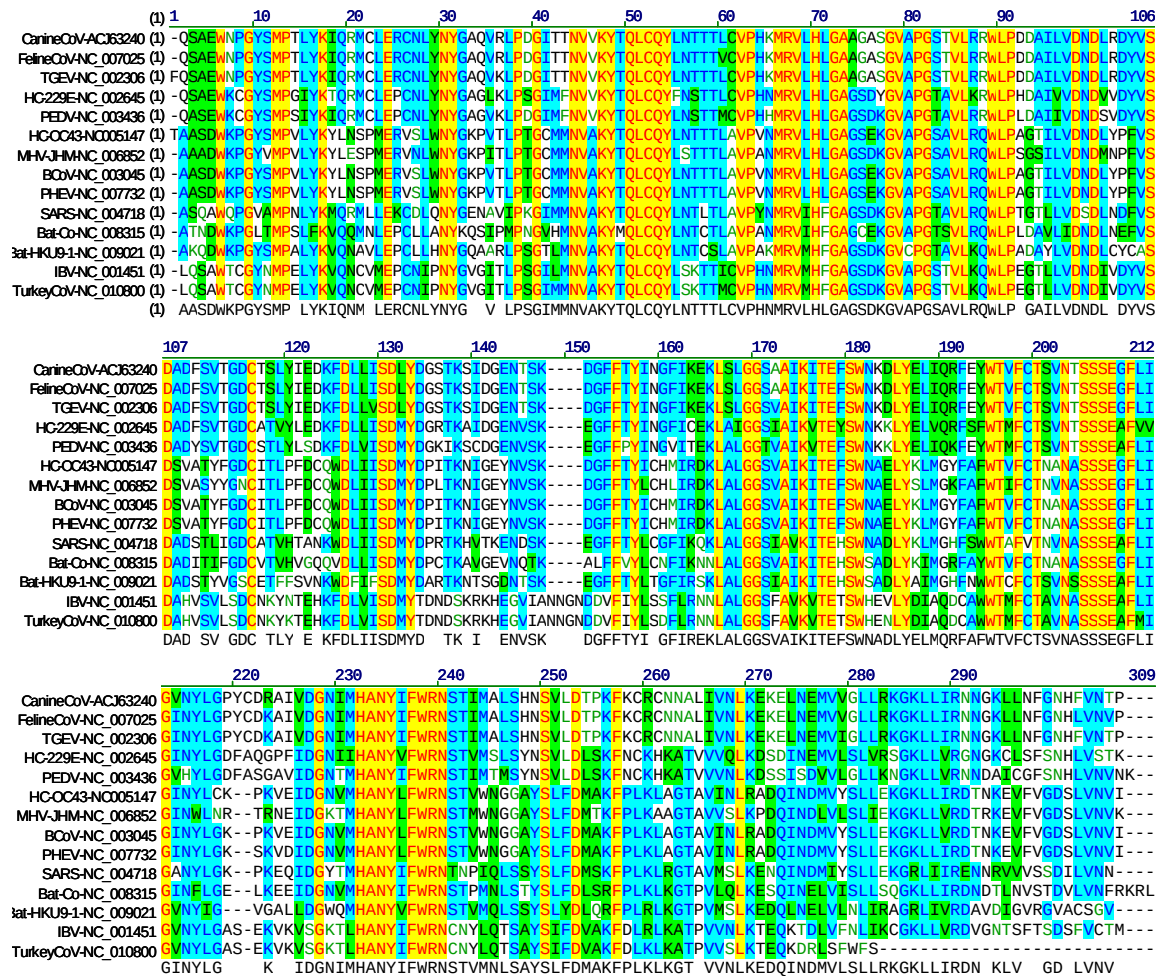


Figure 3. *Mfold*-predicted structures of the 3'-terminal genomic sequences of group 2 CoVs.

BCoV, bovine coronavirus (NC_003045); HC-OC43, human coronavirus OC43 (NC_005147); MHV-JMH, mouse hepatitis virus JMh (NC_006852); Bat-CoV 133/2005, bat coronavirus 133/2005 (NC_008315).

A.



B.

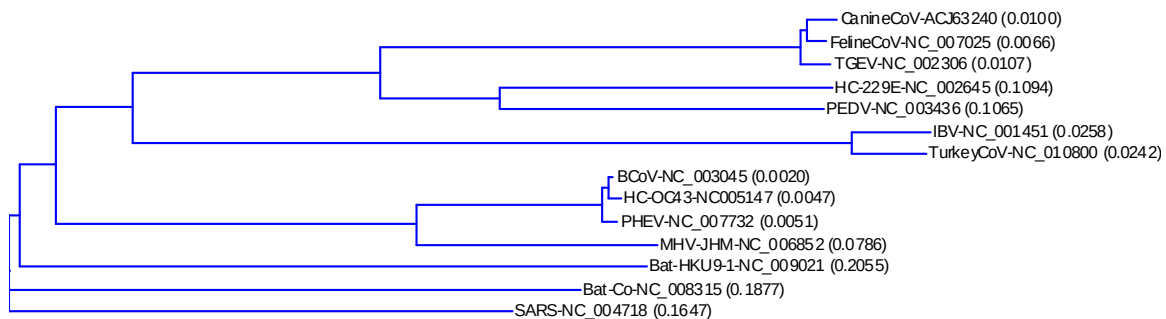


Figure 4. Nsp16 is conserved among all three groups of CoVs.

A. The alignment of CoV nsp16 amino acids representative of all three groups, group 1, 2 and 3 generated with Vector NTI. B. The phylogenetic tree was built using the Neighbor Joining Method.

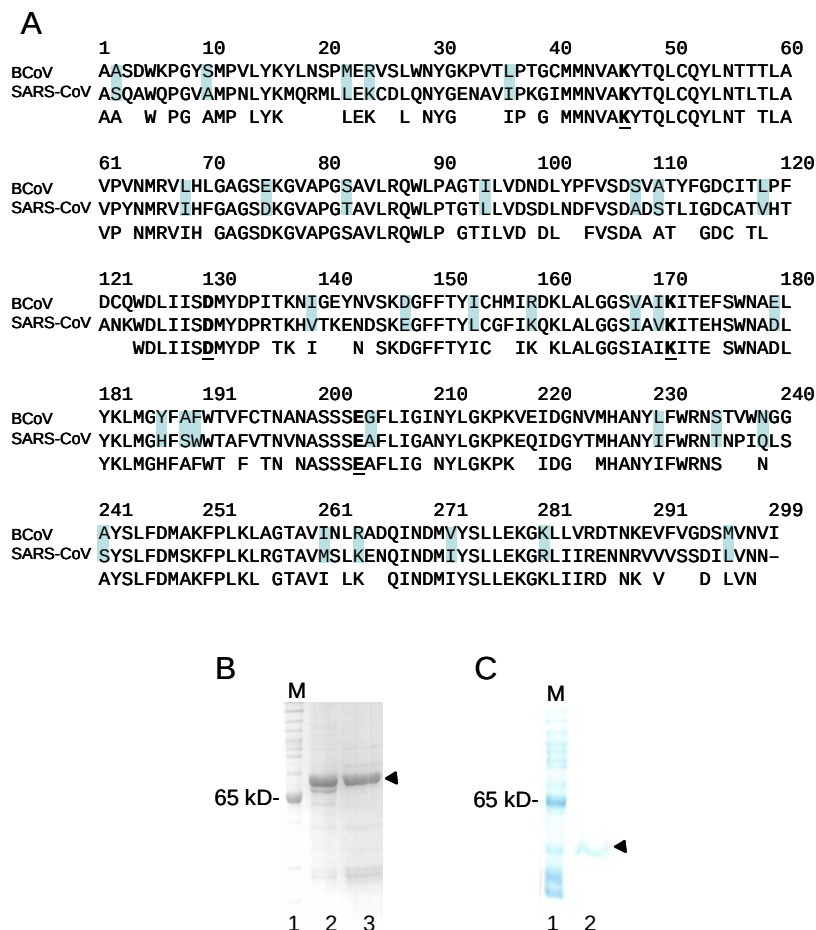


Figure 5. Amino acid sequence and images of purified BCoV 2'-O-MT.

A. Amino acid sequences of the 2'-O-MT from BCoV and SARS-CoV compared. The conserved catalytic active site residues K-D-K-E are underlined. B. GST-BCoV-2'-O-MT fusion protein purified from *E. coli*, resolved by PAGE and stained with Coomassie Blue (pGEX-GST-BCoV-2'-O-MT was cloned by Tara Tucker). The arrowhead identifies the ~65 kDa fusion protein. C. Tag-free BCoV-2'-O-MT expressed from pET-SUMO vector. The arrowhead identifies the ~32 kDa native 2'-O-MT.

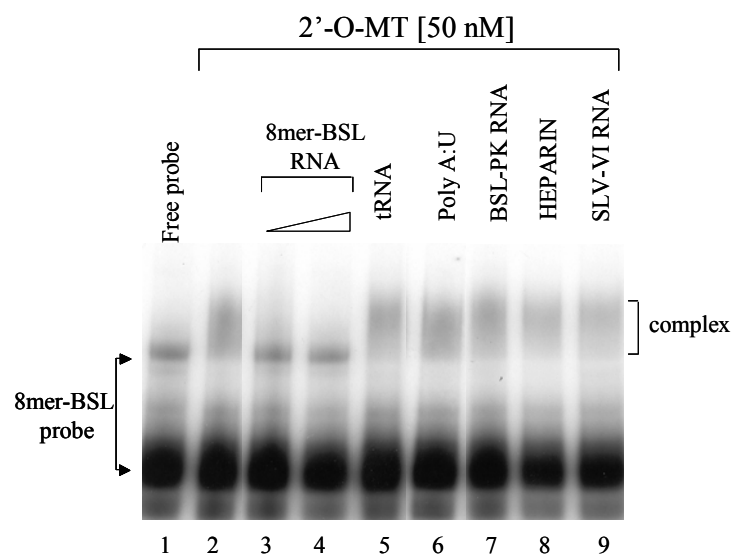


Figure 7. BCoV 2'-O-MT binds 8mer-BSL with specificity.

Formation of a radiolabeled shifted complex (lane 2) is inhibited by the presence of unlabeled 8mer-BSL RNA (lanes 3 and 4), but not by the presence of unlabeled 50 nM tRNA, BSL-PK RNA, poly A: U, heparin, or SLV-VI RNA (lanes 5-9).

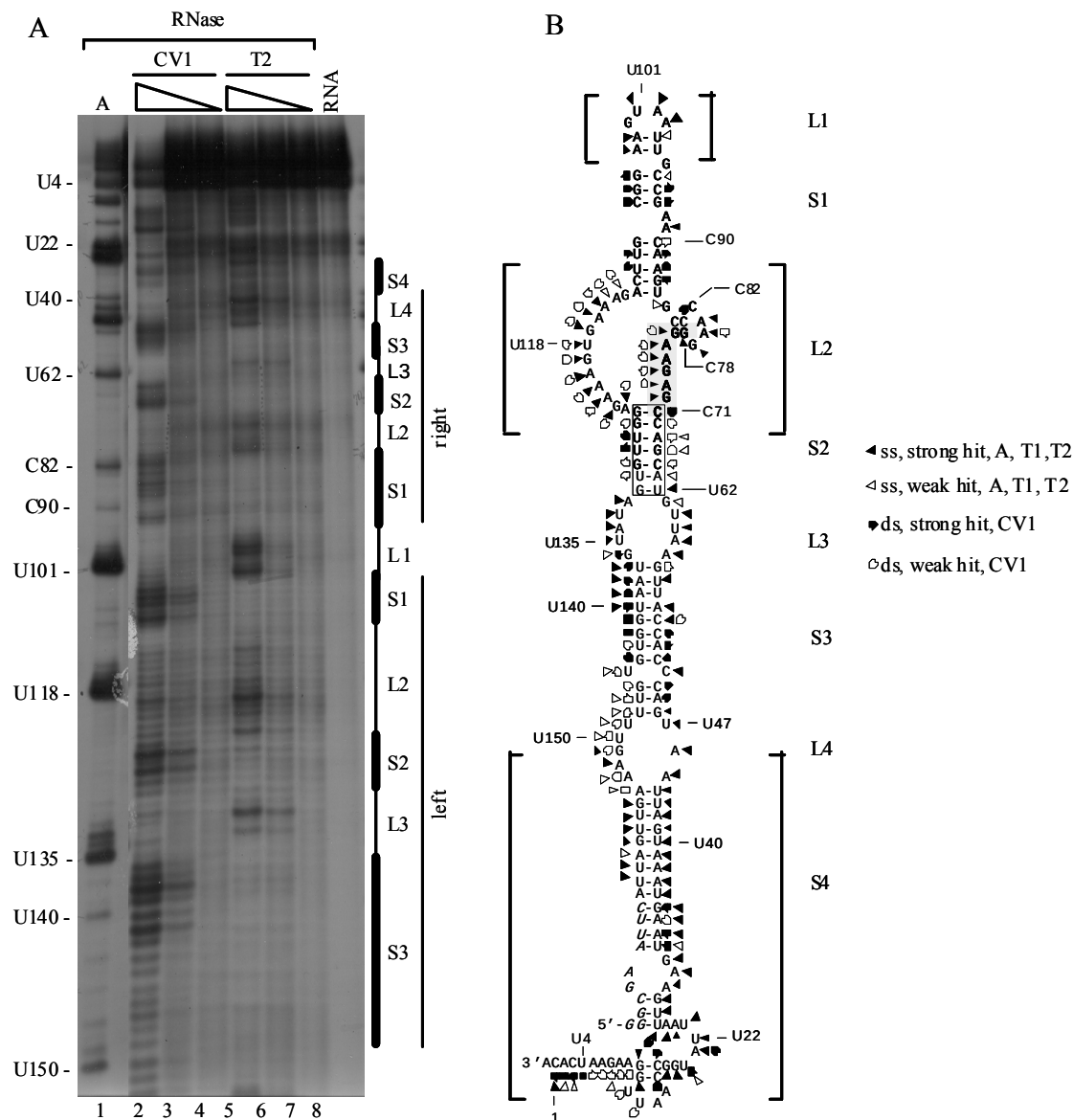


Figure 9. Enzyme structure probing of the BCoV 8mer-BSL.

A. Enzyme probing data. Depicted is a DNA sequencing gel showing separation of 5' end-labeled enzyme-digested products. RNase digestion conditions for the single-strand-digesting RNase T2 and double-strand-digesting RNase CV1 are described in the text. RNA digested with RNase A which cleaves at the 3' end of unpaired C's and U's is shown to identify base positions. Undigested RNA is shown to identify natural breaks in the RNA before RNase treatment. B. Summary of enzyme probing data. The 8mer-BSL as shown has a ΔG of 39.8 kcal/mole.

Figure 10. Footprint analysis of the BCoV 2'-O-MT binding to the 3'-terminal 8mer-BSL.

A. Limited digestion of 5' ^{32} P-end-labeled oct. mut. RNA with the indicated enzymes resolved on a DNA sequencing gel. Lane 1, undigested RNA. Lane 2, alkaline hydrolyzed RNA. Lanes 3, 5, 7 and 9, RNA incubated with enzyme in the absence of 2'-O-MT. Lanes 4, 6, 8 and 10, RNA incubated with enzyme in the presence of 1:90 molar ratio of RNA to 2'-O-MT. B. Same as panel A except for the use of ^{32} P-end-labeled 8mer-BSL RNA. C. Limited digestion of 5' ^{32} P-end-labeled 8mer-BSL with RNase A in the presence of increasing concentrations of 2'-O-MT. Lane 1, undigested RNA. Lane 2, alkaline hydrolyzed RNA. Lane 3, incubation of RNA with RNase A in the absence of 2'-O-MT. Lanes 4, 5 and 6, incubation of RNA with 1:30, 1:60, and 1:90 molar ratios of RNA to 2'-O-MT, respectively. Lane 7, incubation of RNA with 1:90 molar ratio of RNA to GST. D. Summary of regions of RNA protected by 2'-O-MT (unfilled crescents) or made hypersensitive by 2'-O-MT (filled crescents). Unfilled crescents with hatched outline are sites of weak protection.

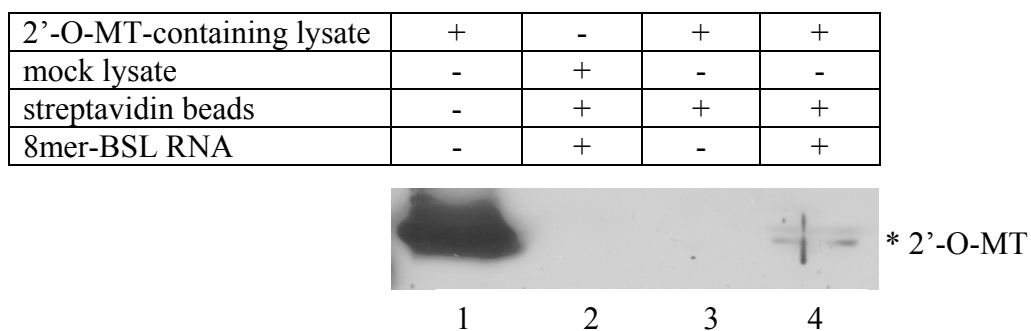


Figure 11. Western analysis of 2'-O-MT expressed *in-vivo* and pulled down with biotinylated 8mer-BSL.

Lane 1, Ni-NTA purified 6xHis-tagged 2'-O-MT over-expressed from DI RNA in BCoV-infected cells. Lane 2, mock-infected, untransfected cell lysate was subjected to Ni-NTA purification and eluate was incubated with streptavidin-prebound 8mer-BSL RNA. Lane 3, 6xHis-tagged 2'-O-MT sample processed as for Lane 1 was incubated with streptavidin beads only. Lane 4, 6xHis-tagged 2'-O-MT sample processed as for Lane 1 was incubated with streptavidin-prebound 8mer-BSL RNA. Each lane represents material from 4×10^6 cells subjected to 12% SDS-PAGE. Western analysis was done with rabbit polyclonal 2'-O-MT-specific antibodies.

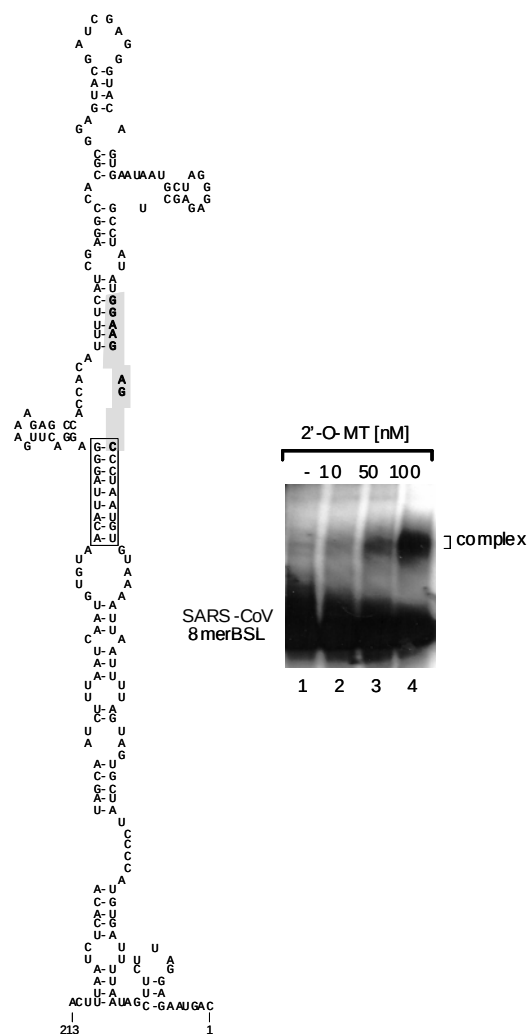


Figure 12. BCoV 2'-O-MT binds the SARS-CoV 8mer-BSL.

A. Mfold-predicted structure for the 8mer-BSL in the SARS-CoV. B. Gel shift of the ^{32}P -radiolabeled SARS-CoV 8mer-BSL with GST-BCoV-2'-O-MT purified from E.coli.

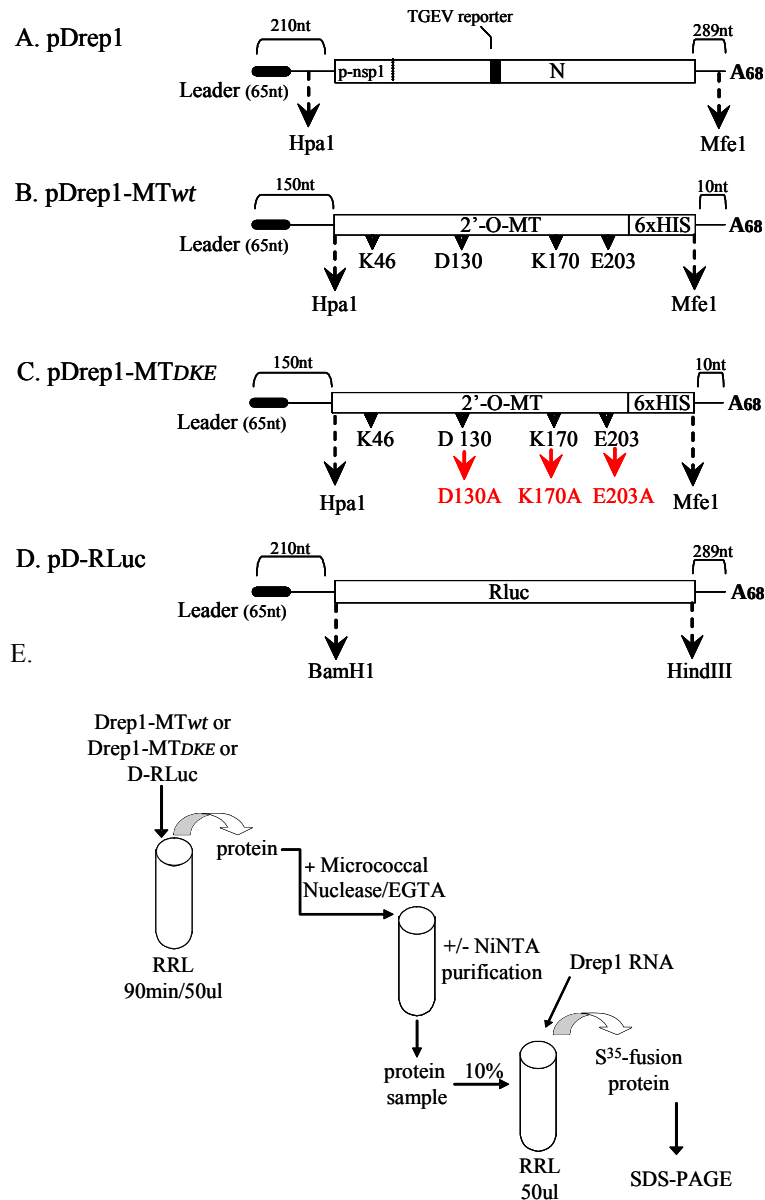


Figure 13. Scheme for testing the effects of *in-vivo* translated 2'-O-MT on translation of the BCoV DI RNA.

A. BCoV DI RNA template translated *in-vitro* to test the effect of added 2'-O-MT on translation. B. Construct for expression of 6xHis-tagged 2'-O-MTwt *in-vitro* for purification. 2'-O-MTwt ORF was cloned into the Hpa1 and Mfe1 restriction sites of pDrep1. C. Construct for expressing 6xHis-tagged 2'-O-MTDKE *in-vitro* containing mutations of 3 active site residues. D. Construct for expressing renilla luciferase (RLuc) used as a control protein in translation experiments. E. Experimental scheme. The pDrep1 fusion protein is ~60kDa, 2'-O-MT protein is ~34kDa and RLuc protein is ~35kDa.

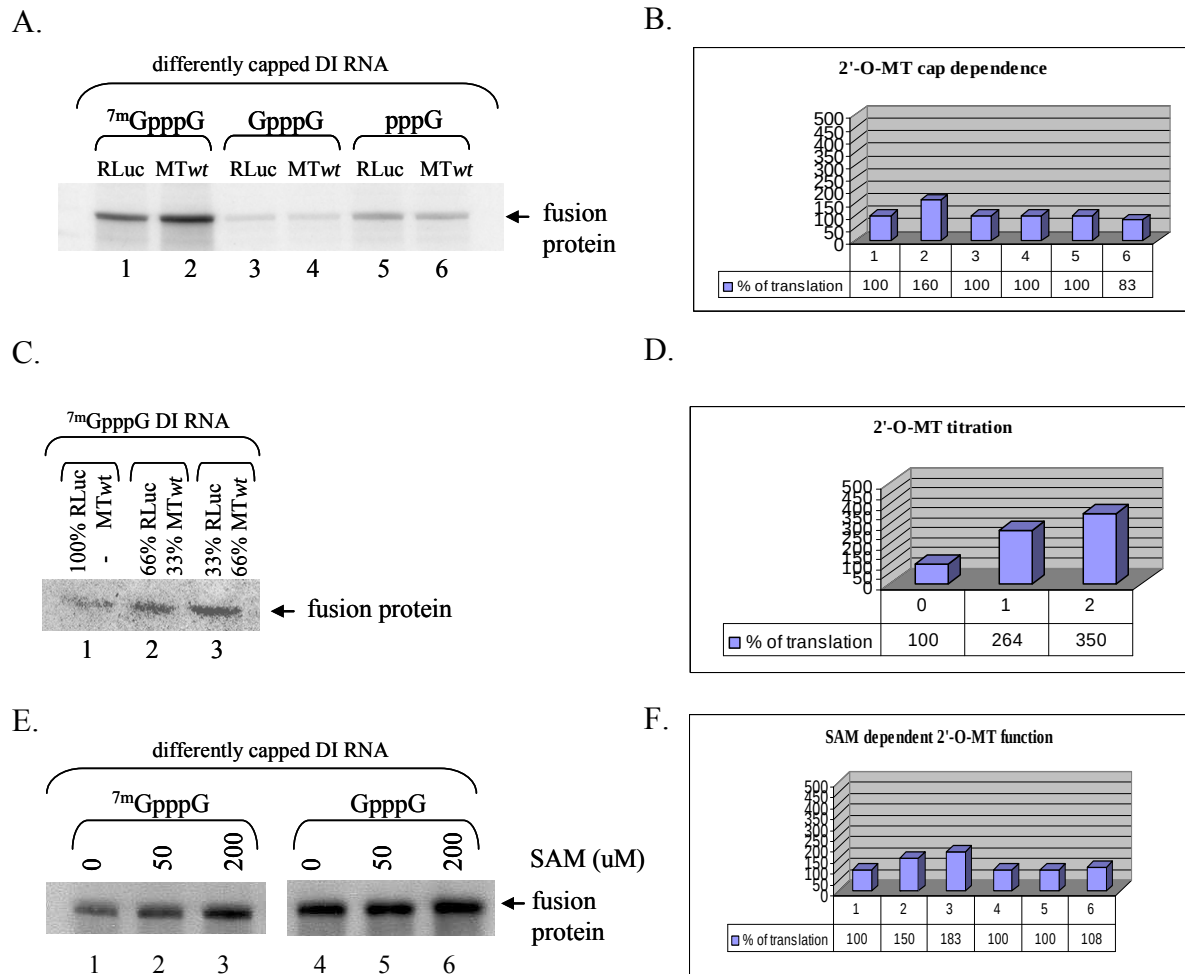


Figure 14. 2'-O-MTwt enhances translation of 7^m GpppG-capped DI RNA *in-vitro* in a SAM dependent manner.

2'-O-MTwt (MTwt) and RLuc proteins were synthesized and purified as described in Figure 13 and used in translation reactions with S^{35} methionine and DI RNA. A. 7^m GpppG-capped, GpppG-capped or uncapped pppG DI RNAs were used as templates. 2'-O-MT enhanced translation of 7^m GpppG-capped DI RNA only. B. ImageJ quantitation of the bands shown in panel A. C. 2'-O-MT translation enhancement increases with increasing concentration of 2'-O-MT. D. ImageJ quantitation of the bands shown in panel C. E. Increase of SAM concentration results in enhancement of 7^m GpppG-capped RNA translation. S^{35} methionine labeled *in-vitro* translation reactions of 7^m GpppG-capped RNA (Lanes: 1-3) and GpppG-capped RNA (Lanes; 4-6). F. ImageJ quantitation of the bands shown at panel E.

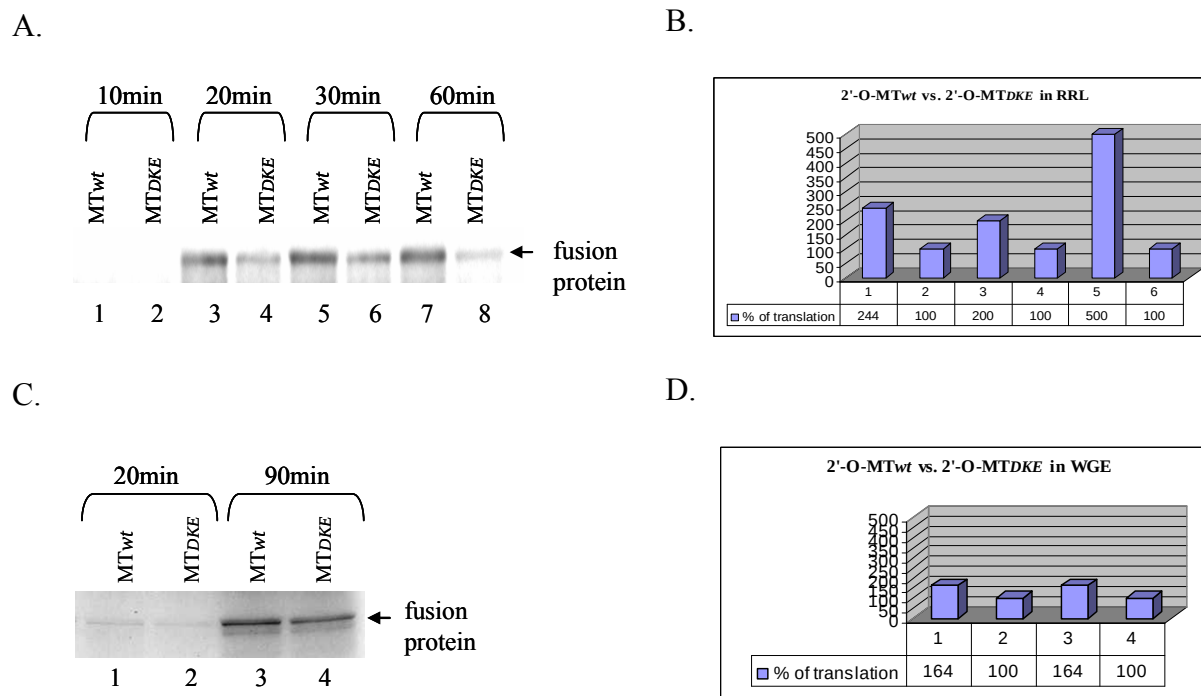


Figure 15. 2'-O-MT with catalytic site mutations does not enhance translation of DI RNA template *in-vitro*.

In-vitro syntheses of 2'-O-MTwt and 2'-O-MTDKE were performed according to the manufacturer's protocol. No enhancement of Drep1 translation was observed in the Rabbit Reticulocyte Lysate (RRL) (A, B) or in the Wheat Germ Extract System (WGE) (C, D) when mutated 2'-O-MT was added to the reaction with ^{7m} GpppG-capped Drep1 RNA as a template.

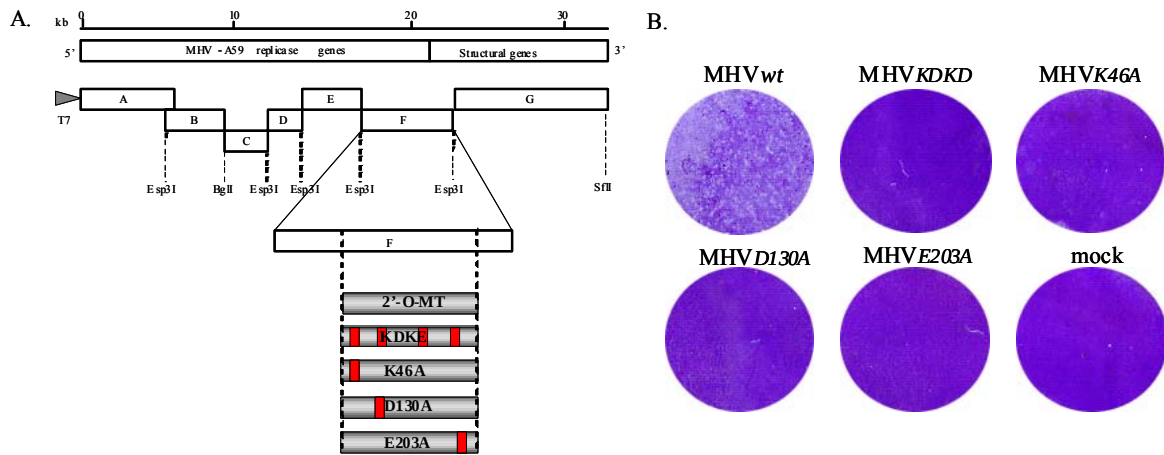
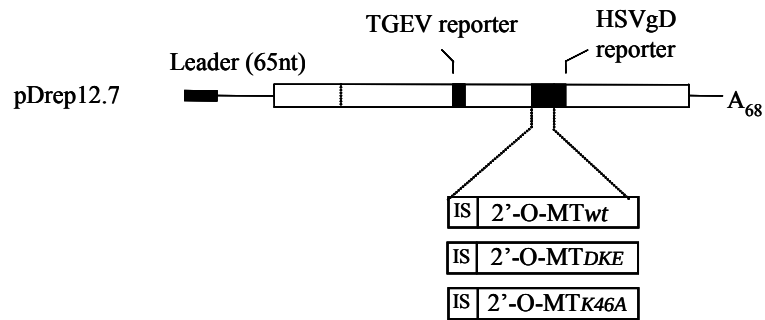


Figure 17. Assembly of an MHV-A59 full-length infectious clone with wt 2'-O-MT and with 2'-O-MT with mutated catalytic active sites.

A. MHV fragments A through G and the engineered restriction sites as designed by R. Baric et al. The 2'-O-MT cistron is localized within the F fragment at nt 5096-5992. 2'-O-MT catalytic active site residues were replaced by alanine to generate 4 mutants K46A-D130A-K170A-E203A, K46A, D130A, and E203A. B. Cytopathology as observed on DBT cells at 72 hr post-transfection with MHV wild type and mutant recombinants as noted.

A.



B.

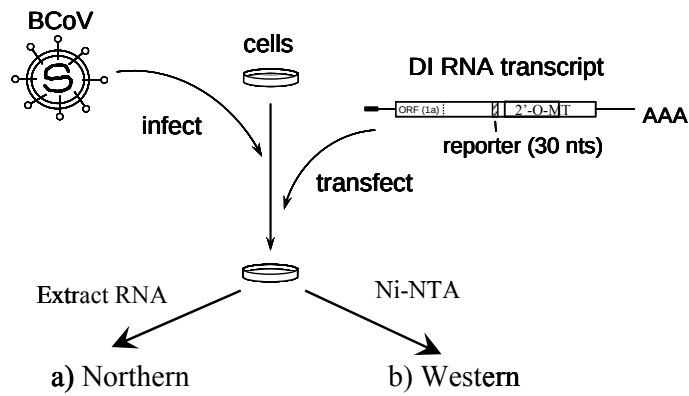
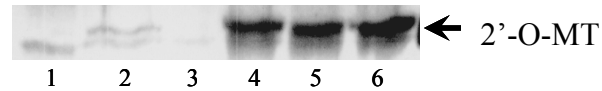


Figure 18. BCoV 2'-O-MT over-expression system.

A. Constructs used for 2'-O-MT over-expression experiments: pDrep12.7, pDrep12.7-MT_{wt}, pDrep12.7-MT_{DKE} and pDrep12.7-MT_{K46A}. IS, intergenic sequence. B. Experimental scheme.

A.



B.

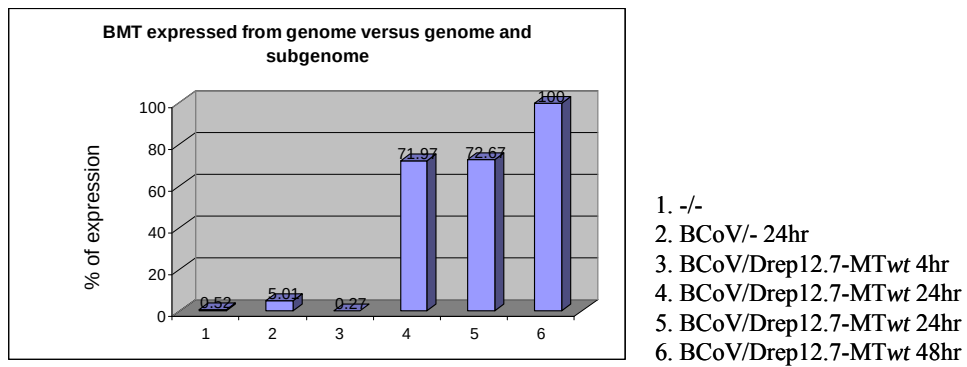


Figure 19. BCoV 2'-O-MTwt is over-expressed from Drep12.7-MTwt.

A. Western analyses of cellular extracts. Lane 1, uninfected HRT cells; Lane 2, BCoV-infected HRT cells; Lanes 3 through 6, BCoV-infected and Drep12.7-MTwt transfected cells harvested at the times indicated. For Western analysis, BCoV 2'-O-MT specific antibodies were used. B. Summary of ImageJ analysis. ImageJ analysis of the Western data indicates ~60 fold over-expression of 2'-O-MT in lanes 4-6.

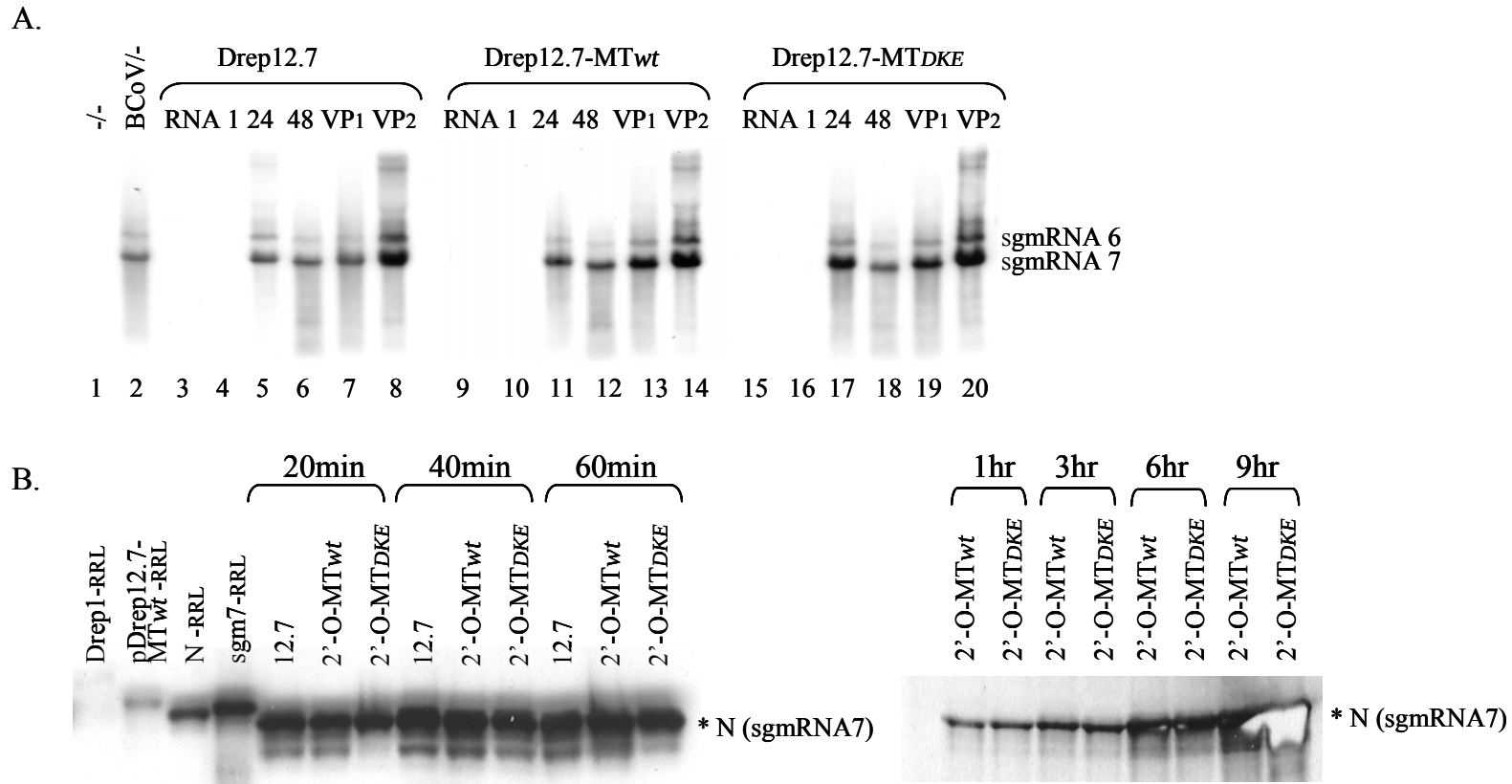


Figure 20. 2'-O-MT over-expression has no effect on virus replication and sgRNAs translation *in-vivo*.

A. Northern analysis of viral RNA accumulation upon over-expression of 2'-O-MTwt and 2'-O-MTDKE. The blot was probed with N protein-specific probe (N(+)). Subgenomic mRNAs 6 and 7 are noted. B. Western analysis of N protein expression from sgRNA7 with N protein specific antibodies. Cell lysates of cells transfected with constructs Drep12.7, Drep12.7-MTwt and Drep12.7-MTDKE at indicated times post-transfection were precipitated with ammonium sulfate prior to SDS-PAGE and Western analysis.

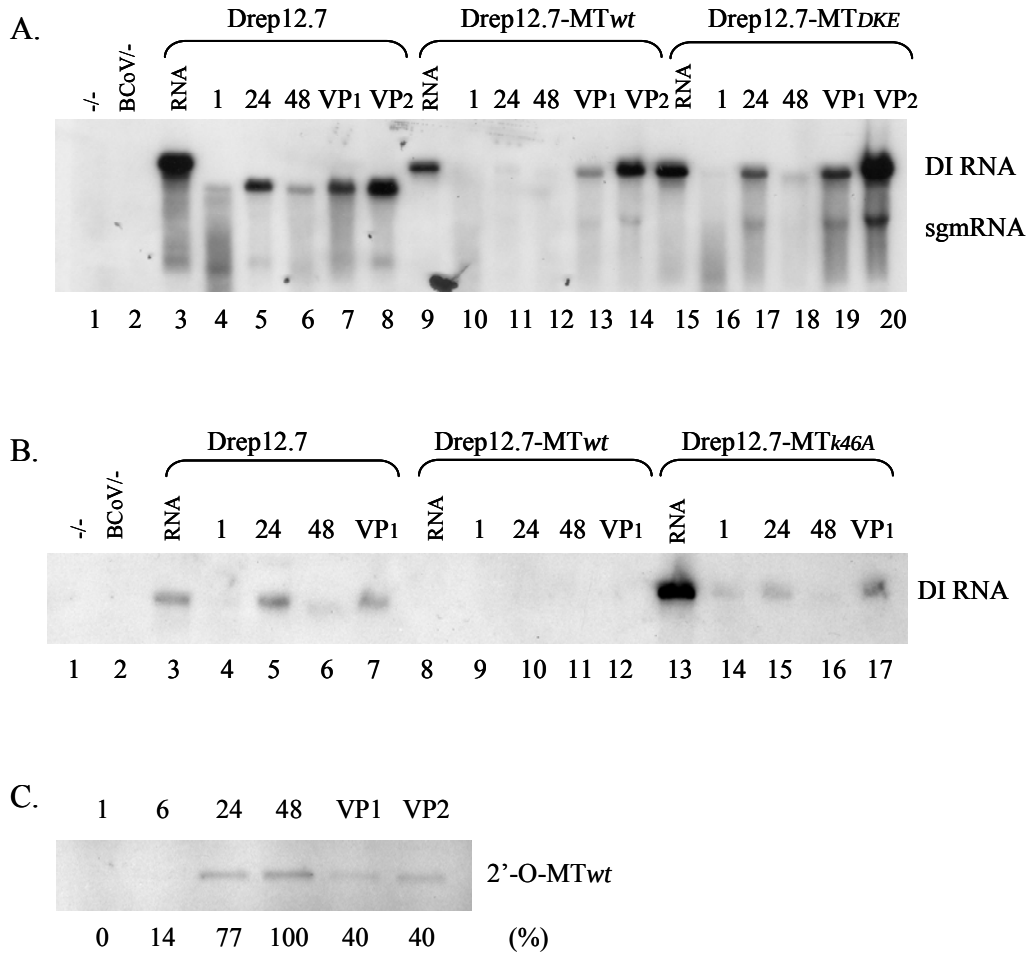
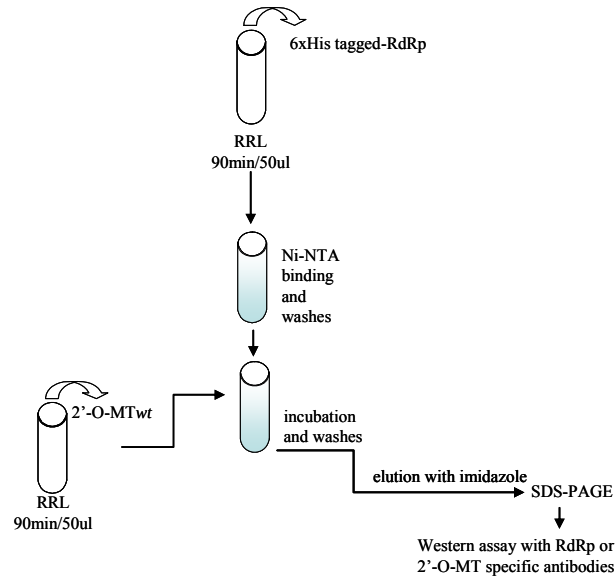


Figure 21. Effect of 2'-O-MT over-expression on the accumulation of DI RNA.

A. Northern analysis of Drep12.7-MTwt and Drep12.7-MTDKE replication probed with HSVgD (+) probe. 2'-O-MT sgmRNA was expressed from DI RNA in all tested constructs. The effect of the 2'-O-MTwt and 2'-O-MTDKE mutant over-expression on DI RNA replication was evaluated in comparison to Drep12.7. B. Northern analysis of Drep12.7-MTwt and Drep12.7-MTK46A. C. Western analysis of 2'-O-MTwt expressed from sgmRNA at different times post-transfection.

A.



B.

6xHis RdRp	+	+	+
RRL mix	-	+	-
2'-O-MTwt	+	-	+
RdRp Ab	+	-	-
2'-O-MT Ab	-	+	+

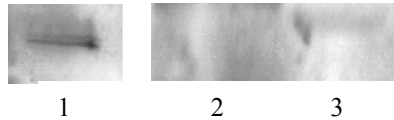


Figure 22. *In-vitro* synthesized 6xHis-tagged RdRp pulls-down *in-vitro* synthesized 2'-O-MT.

A. *In-vitro* synthesized RdRp with C-terminal 6xHis Tag was bound to Ni-NTA and incubated with *in-vitro* synthesized 2'-O-MT on Ni-NTA columns. Imidazole eluted protein complexes were analyzed by 12% SDS-PAGE followed by Western analysis with both RdRp specific and 2'-O-MT specific antibodies. B. Lane 1; Ni-NTA elute of 6xHis-tagged RdRp incubated with *in-vitro* synthesized 2'-O-MT blotted with RdRp specific antibodies. Lane 2; Ni-NTA elute of 6xHis-tagged RdRp incubated with RRL mix blotted with 2'-O-MT specific antibodies. Lane 3; Ni-NTA elute of 6xHis-tagged RdRp incubated with *in-vitro* synthesized 2'-O-MT blotted with 2'-O-MT specific antibodies.

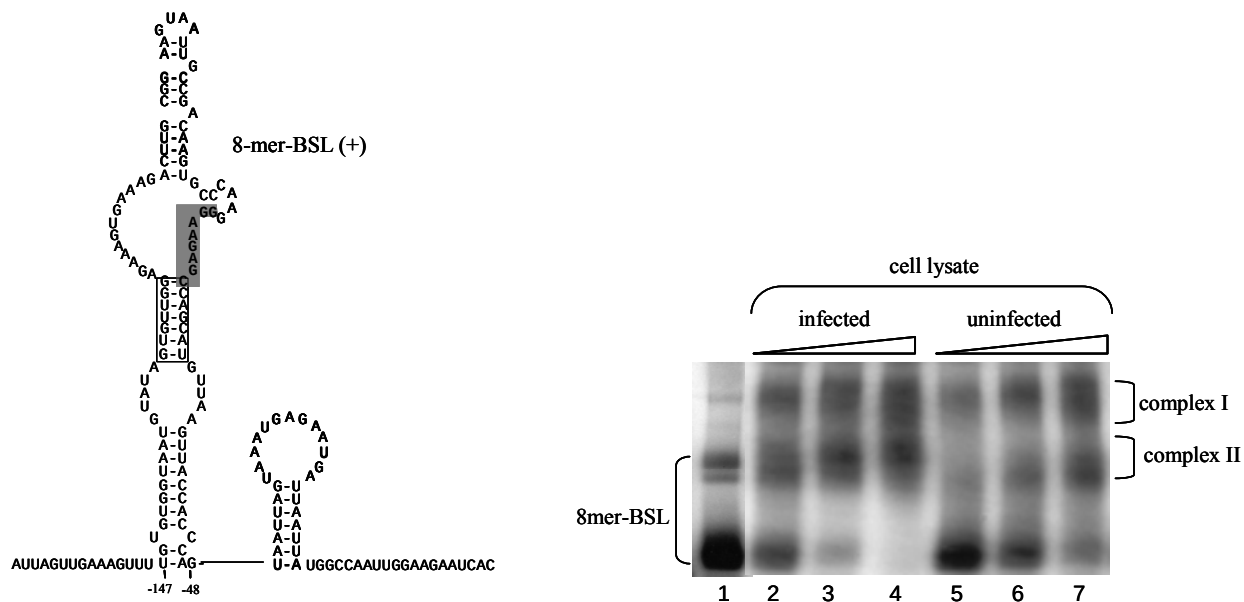


Figure 24. Gel shift of the BCoV 8mer-BSL by proteins from uninfected and infected cells. Cell lysates used were made from uninfected or BCoV-infected HRT cells. 5, 10, 20 ug of proteins in cell lysates were used per reaction. One cellular protein complex was formed with the 8mer-BSL probe.

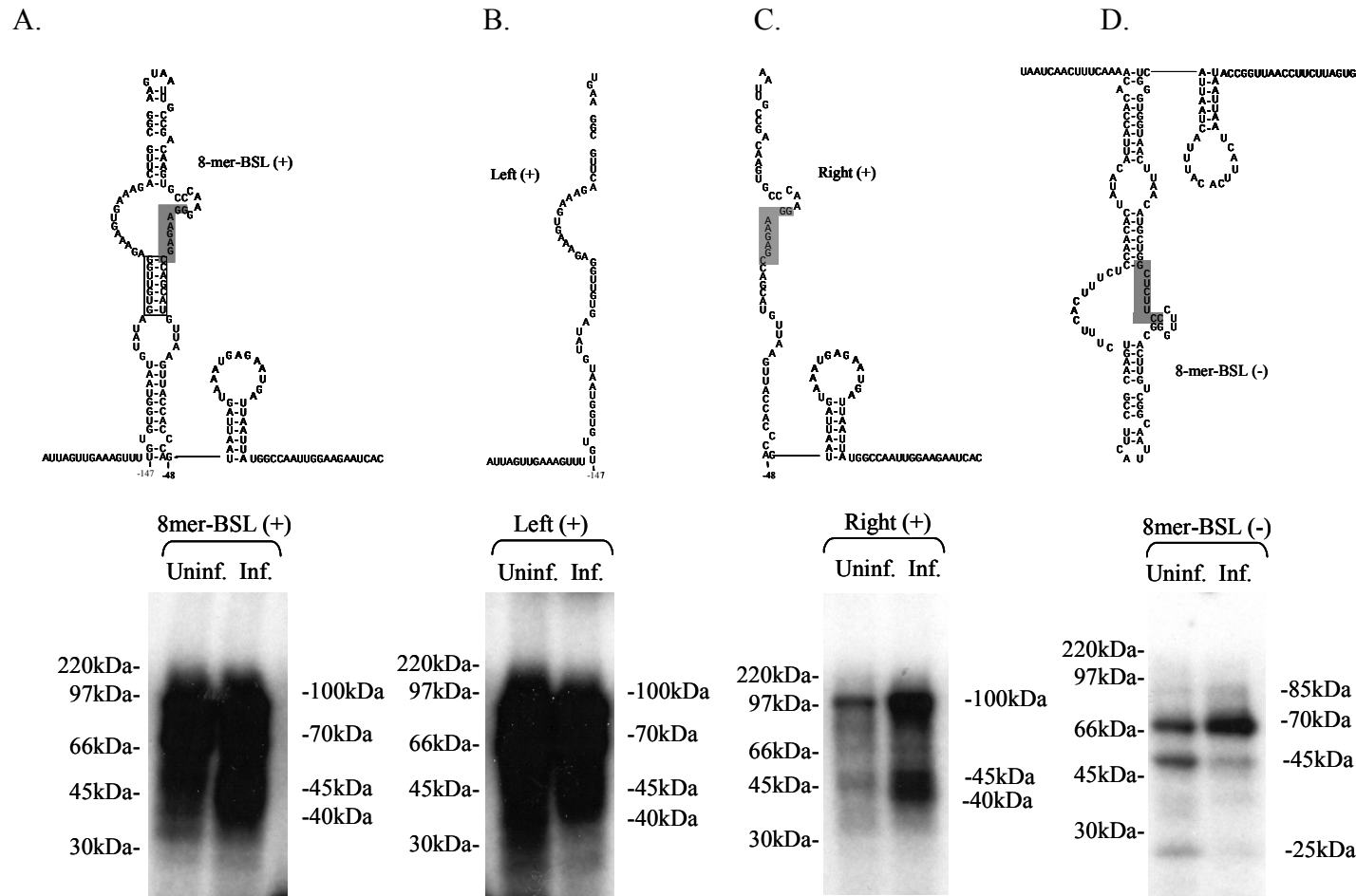


Figure 25. Cellular and viral proteins that UV-cross-link to the BCoV 8mer-BSL.

A. Three cellular and one viral protein UV-cross-link to the full length 8mer-BSL probe in (+) strand. B. Three cellular and one viral protein UV-cross-links to the left half of the 8mer-BSL probe. C. Two cellular and one viral protein bind the right half of the 8mer-BSL. D. Four cellular proteins bind the (-) strand of the 8mer-BSL. Uninf., uninfected cell lysate; Inf., infected cell lysate.

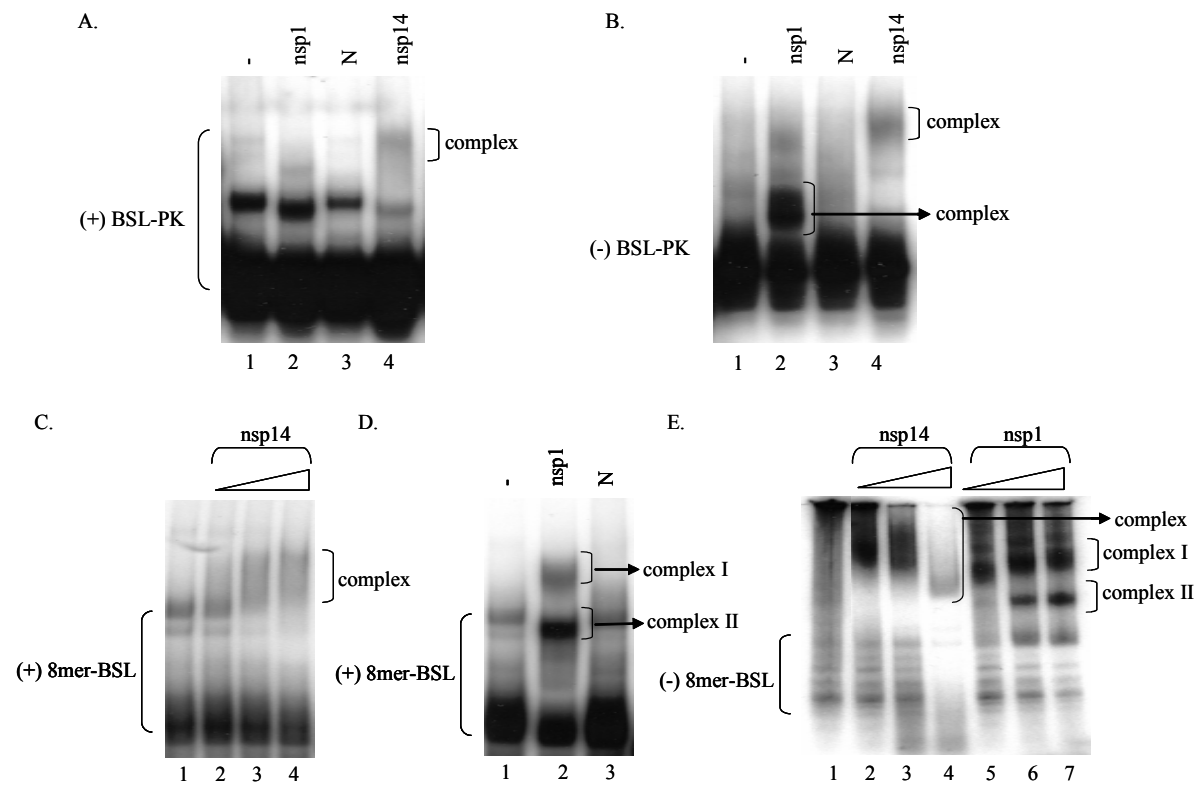


Figure 26. Gel shift of probes representing (+) and (-) strand forms of the BCoV BSL-PK and 8mer-BSL with E.coli expressed viral proteins nsp1, N and nsp14.

A. The positive-strand BSL-PK interacts with nsp14 (lane 4), but not with nsp1 (lane 1) or N protein (lane 3). B. The negative-strand BSL-PK interacts with nsp1 (lane 1) and nsp14 (lane 4), but not with N protein (lane 3). C. The positive-strand 8mer-BSL interacts with nsp14. D. The positive strand 8mer-BSL interacts with nsp1 (lane 2) but not with N protein (lane 3). E. The negative-strand 8mer-BSL interacts with nsp14 (lanes 2-3) and with nsp1 (lanes 5-7). The molar ratio of probe to protein used was 1:430 for nsp1 (p28), 1:140 for N protein and 1:750 for nsp14 (exonuclease).

mock	+	-	-	-	-	-	-
2'-O-MT <i>in-vivo</i>	-	+	+	+	+	-	-
2'-O-MT <i>in-vitro</i>	-	-	-	-	-	+	+
CIP	-	-	+	-	+	-	+
EDTA	-	+	+	-	-	-	-

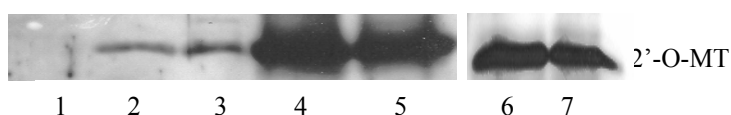


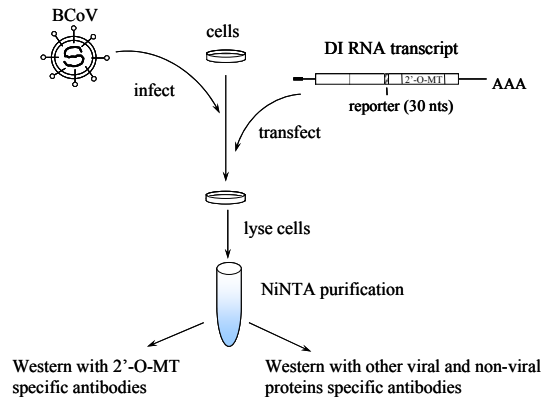
Figure 27. Phosphorylation status of the BCoV 2'-O-MT.

6xHis-tagged 2'-O-MT over-expressed and purified from HRT cells and 6xHis-tagged 2'-O-MT synthesized *in-vitro* were analyzed by Western assay with 2'-O-MT specific antibodies. *In-vivo* 2'-O-MT preparations purified with EDTA in lysis buffer (lanes 2 and 3) and purified without EDTA in lysis buffer (lanes 4 and 5). Each preparation was treated with calf intestinal phosphatase (CIP) (lanes 3 and 5). CIP untreated sample was used as a control (lanes 2 and 4). Lanes 6 and 7- Western analysis of 2'-O-MT translated *in-vitro* in RRL with (lane 7) or without (lane 6) CIP treatment. No changes in mobility were observed as a function of phosphatase treatment suggesting 2'-O-MT is not highly phosphorylated. Low level phosphorylation cannot be ruled out from this analysis.

Figure 28. BCoV 2'-O-MT binds both N and nsp1 proteins *in-vivo*.

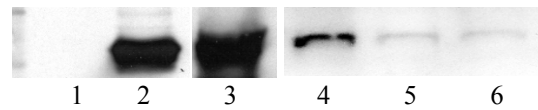
In-vivo expressed 6xHis-tagged 2'-O-MT was pulled-down by nickel affinity chromatography and analyzed with Western Assay with specific antibodies. A. Experimental scheme. B. Binding of N protein to 2'-O-MT. Lane 1; Ni-NTA purified protein preparations from uninfected, untransfected cell lysates. Lane 2; Ni-NTA pulled-down 2'-O-MT from BCoV-infected, pDrep12.7-MTwt transfected cells blotted with N-specific antibodies. Lanes 3 and 4; Ni-NTA pulled down 2'-O-MT untreated and micrococcal nuclease treated samples, respectively. Lanes 5, 6, 7: Ni-NTA purified 2'-O-MT washed with increasing NaCl prior to imidazole elution. C. Binding of nsp1 to 2'-O-MT. Lane 1; Ni-NTA purified protein preparations from uninfected, untransfected cell lysates. Lane 2, Ni-NTA pulled-down 2'-O-MT from BCoV-infected, pDrep12.7-MTwt transfected cells blotted with nsp1-specific antibodies. Lanes 3 and 4; Ni-NTA purified 2'-O-MT untreated and micrococcal nuclease treated, respectively. Lanes 5, 6, 7; Ni-NTA pulled-down 2'-O-MT washed with increasing NaCl prior to imidazole elution.

A.



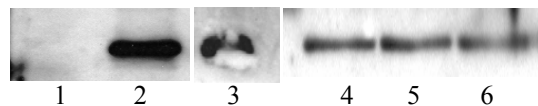
B. 2'-O-MT weakly binds N protein *in-vivo*.

uninfected, untransfected	+	-	-	-	-	-
BCoV-infected, pDrep12.7-MTwt transfected	-	+	+	+	+	+
micrococcal nuclease	-	-	+	-	-	-
150 mM NaCl	+	+	+	+	-	-
300 mM NaCl	-	-	-	-	+	-
450 mM NaCl	-	-	-	-	-	+
Western with N-specific antibody						



C. 2'-O-MT strongly binds nsp1 protein *in-vivo*.

uninfected, untransfected	+	-	-	-	-	-
BCoV-infected, pDrep12.7-MTwt transfected	-	+	+	+	+	+
micrococcal nuclease	-	-	+	-	-	-
150 mM NaCl	+	+	+	+	-	-
300 mM NaCl	-	-	-	-	+	-
450 mM NaCl	-	-	-	-	-	+
Western with nsp1-specific antibody						



VITA

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