Bovine Coronavirus Nonstructural Protein 1 (p28) Is an RNA Binding Protein That Binds Terminal Genomic cis-Replication Elements

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Nonstructural protein 1 (nsp1), a 28-kDa protein in the bovine coronavirus (BCoV) and closely related mouse hepatitis coronavirus, is the first protein cleaved from the open reading frame 1 (ORF 1) polyprotein product of genome translation. Recently, a 30-nucleotide (nt) cis-replication stem-loop VI (SLVI) has been mapped at nt 101 to 130 within a 288-nt 5′-terminal segment of the 738-nt nsp1 cistron in a BCoV defective interfering (DI) RNA. Since a similar nsp1 coding region appears in all characterized groups 1 and 2 coronavirus DI RNAs and must be translated in cis for BCoV DI RNA replication, we hypothesized that nsp1 might regulate ORF 1 expression by binding this intra-nsp1 cistronic element. Here, we (i) establish by mutation analysis that the 72-nt intracistronic SLV immediately upstream of SLVI is also a DI RNA cis-replication signal, (ii) show by gel shift and UV-crosslinking analyses that cellular proteins of ~60 and 100 kDa, but not viral proteins, bind SLV and SLVI, (SLV-VI) and (iii) demonstrate by gel shift analysis that nsp1 purified from Escherichia coli does not bind SLV-VI but does bind three 5′-untranslated region (UTR)- and one 3′ UTR-located cis-replication SLs. Notably, nsp1 specifically binds SLIII and its flanking sequences in the 5′ UTR with ~2.5 μM affinity. Additionally, under conditions enabling expression of nsp1 from DI RNA-encoded subgenomic mRNA, DI RNA levels were greatly reduced, but there was only a slight transient reduction in viral RNA levels. These results together indicate that nsp1 is an RNA-binding protein that may function to regulate viral genome translation or replication but not by binding SLV-VI within its own coding region.

Coronaviruses (CoVs) (59) cause primarily respiratory and gastroenteric diseases in birds and mammals (35, 71). In humans, they most commonly cause mild upper respiratory disease, but the recently discovered human CoVs (HCoVs), HCoV-NL63 (65), HCoV-HKU1 (73), and severe acute respiratory syndrome (SARS)-CoV (40) cause serious diseases in the upper and lower respiratory tracts. The SARS-CoV causes pneumonia with an accompanying high (~10%) mortality rate (69). The ~30-kb positive-strand CoV genome, the largest known among RNA viruses, is 5′ capped and 3′ polyadenylated and replicates in the cytoplasm (41). As with other characterized cytoplasmically replicating positive-strand RNA viruses (3), translation of the CoV genome is an early step in replication, and terminally located cis-acting RNA signals regulate translation and direct genome replication (41). How these happen mechanistically in CoVs is only beginning to be understood.

In the highly studied group 2 mouse hepatitis coronavirus model (MHV A59 strain) and its close relative the bovine CoV (BCoV Mebus strain), five higher-order cis-replication signals have been identified in the 5′ and 3′ untranslated regions (UTRs). These include two in the 5′ UTR required for BCoV defective interfering (DI) RNA replication (Fig. 1A) described as stem-loop III (SLIII) (50) and SLIV (51). Recently, the SLI region in BCoV (15) has been reanalyzed along with the homologous region in MHV and is now described as comprising SL1 and SL2 (Fig. 1A), of which SL2 has been shown to be a cis-replication structure in the context of the MHV genome (38). In the 3′ UTR, two higher-order cis-replication structures have been identified that function in both DI RNA and the MHV genome. These are a 5′-proximal bulged SL and adjacent pseudoknot that potentially act together as a unit (23, 27, 28, 72) and a 3′-proximal octamer-associated bulged SL (39, 76) (Fig. 1A). In addition, the 5′-terminal 65-nucleotide (nt) leader and the 3′-terminal poly(A) tail have been shown to be cis-replication signals for BCoV DI RNA (15, 60).

In CoVs, the 5′-proximal open reading frame (ORF) of ~20 kb (called ORF 1) comprising the 5′-two-thirds of the genome is translated to overlapping polyproteins of ~500 and ~700 kDa, named pp1a and pp1ab (41). pp1ab is formed by a ~1 ribosomal frameshift event at the ORF1a-ORF1b junction during translation (41). pp1a and pp1ab are proteolytically processed into potentially 16 nonstructural protein (nsp) end products or partial end products that are proposed to function together as the replicase (24). ORF 1a encodes nsps 1 to 11 which include papain-like proteases (nsp3), a 3C-like main protease (nsp5), membrane-anchoring proteins (nsps 4 and 6), a potential primase (nsp8), and RNA-binding proteins (nsp 7/nsp 8 complex and nsps 9 and 10) of imprecisely understood function (19, 20, 24, 25, 29, 43, 49, 77). ORF 1b encodes nsps 12 to 16 which function as an RNA-dependent RNA polymerase, a helicase, an exonuclease, an endonuclease, and a 2′-O-methyltransferase, respectively (6, 17, 24, 44). 3′ Proximal genomic ORFs encoding structural and accessory proteins are translated from a 3′-nested set of subgenomic mRNAs (sgm-RNAs) (41).
The N-terminal ORF 1a protein, nsp1, in the case of BCoV and MHV is also named p28 to identify the cleaved 28-kDa product (18). The precise role of nsp1 in virus replication has not been determined, but it is known that a sequence encoding an N-proximal nsp1 region in MHV (nt 255 to 369 in the 738-nt coding sequence) cannot be deleted from the genome without loss of productive infection (10). nsp1 also directly binds nsp7 and nsp10 (11) and by confocal microscopy is found associated with the membranous replication complex (10, 66) and virus assembly sites (11). The amino acid sequence of nsp1 is poorly conserved among CoVs, indicating that it may be a protein that interacts with cellular components (1, 58). In the absence of other viral proteins, MHV nsp1 induces general host mRNA degradation (79) and cell cycle arrest (16). The SARS-CoV nsp1 homolog, a 20-kDa protein, has been reported to cause mRNA degradation (30, 45), inhibition of host protein synthesis (30, 45, 70), inhibition of interferon signaling (70, 79), and cytokine dysregulation in lung cells (36).

In this study, we examine the RNA-binding properties of BCoV nsp1 with the hypothesis that it is a potential regulator of translation or replication through its binding of SLVI mapping within its coding region. The rationale for this hypothesis stems from five observations. (i) In the BCoV DI RNA, the 5′-terminal one-third (approximately) of the nsp1 cistron and the entire nucleocapsid (N) protein cistron together comprise the single contiguous ORF in the DI RNA, and most of both coding regions appear required for DI RNA replication (15). (ii) A cis-acting SL named SLVI is found within the partial nsp1 cistron in the BCoV DI RNA (12). (iii) A similar part of the nsp1 cistron is found in the genome of all characterized naturally occurring group 1 and 2 CoV DI RNAs described to date (7, 8). (iv) A cis-acting SL named SLVI is found within the partial nsp1 cistron in the BCoV DI RNA (12). (v) Translation, which involves a 5′→3′ transit of ribosomes, and negative-strand synthesis, which involves a 3′→5′ transit of the RNA-dependent RNA polymerase, cannot simultaneously occur on the same molecule with a single ORF (4, 31). Thus, to enable genome replication an inhibition of translation at least early in infection for cytoplasmically replicating positive-strand RNA viruses is required (4, 5, 22, 32). Mechanisms of translation inhibition have been described for the QB viral genome, wherein the viral replicase autoregulates translation by binding an intracistronic cis-replication element (32), and for the polio virus genome, wherein genome circularization inhibits the early translation step (5, 22). Therefore, since nsp1 is synthesized early and also contains an intracistronic cis-replication element, we postulated that it is autoregulatory with RNA binding properties.

Here, we do the following: (i) demonstrate by mutagenesis analysis that the 72-nt SLV, mapping immediately upstream of SLVI and within the partial nsp1 cistron, is also a cis-acting DI RNA replication element; (ii) show by gel shift and UV cross-linking analyses that there is likely no binding of an intracellular viral protein to SLV and SLVI (SLV-VI), but there is binding of unidentified cellular proteins of ~60 and 100 kDa; and (iii) show by gel shift analysis that recom-

![Figure 1](https://jvi.asm.org/FIG. 1. RNA structures in the BCoV genome tested for nsp1 binding. (A) BCoV 5′-terminal and 3′-terminal cis-acting RNA SL structures and flanking sequences identified for BCoV DI RNA replication. Regions of the genome are identified and SL cis-replication elements are identified schematically. Open boxes at nt 100 and 211 identify AUG start codons for the short upstream ORF and ORF 1, respectively. A closed box at nt 124 identifies the UAG stop codon for the short upstream ORF. Shown below the SL structures are the RNA segments used as 32P-labeled probes in the gel shift assays. BSL-PK, bulged SL-pseudoknot; 8mer-BSL, octamer-associated bulged SL. (B) Gel shift assays for probes when used with purified nsp1. Protein-RNA complexes identifying a shifted probe are labeled C.)
binant nsp1 purified from *Escherichia coli* does not bind SLV-VI but does bind SLs I to IV in the 5′ UTR and also the 3′-terminal bulged SL in the 3′ UTR, suggesting a possible regulatory role at these sites. Notably, specific binding is observed at these sites. While SLV-VI but does bind SLs I to IV in the 5′ UTR, suggesting a possible regulatory role at these sites. Notably, specific binding is observed at these sites.

**MATERIALS AND METHODS**

**Cells and viruses.** A DI RNA-free stock of the Mebus strain of BCoV (GenBank accession number U00735) at 4.5 × 10⁶ PFU/ml (14, 15) was grown on human rectal tumor cell line HRT-18 (64) as described previously (56, 75). Briefly, cells (10⁶) at 80% confluence in a 35-mm dish were infected with BCoV at a multiplicity of 10 PFU per cell and transfected 1 h later with 500 ng of RNA using Lipofectin (Invitrogen) and 300 Ci of [3²P]UTP (3,000 Ci/m mole) (MP Biochemicals). The mixture was treated with 3 U of RQ1 RNase-free DNase (Promega). RNA was purified by silica matrix affinity using an RNaid kit (MP Biochemicals) and quantified spectrophotometrically.

**RNA synthesis.** For synthesis of uncapped positive-strand transcripts of SLV DI RNA mutants for testing mutational effects on DI RNA replication, 2 μg of Mvul-linearized plasmid DNA was transcribed in a 100-μl reaction mixture containing 40 U of T7 RNA polymerase (Promega) following the Promega protocol for synthesis of uncapped nonlabeled RNA. The mix was treated with 3 U of RQ1 RNase-free DNase (Promega), and RNA was purified by silica matrix affinity using an RNaid kit (MP Biochemicals) and quantified spectrophotometrically.

**Northern assay for DI RNA replication.** Northern blot assays for detecting reporter-containing DI RNAs were performed essentially as described previously (56, 75). Briefly, cells (∼6 × 10⁶) at ∼80% confluence in a 35-mm dish were infected with BCoV at a multiplicity of 10 PFU per cell and transfected 1 h later with 500 ng of RNA using Lipofectin (Invitrogen). At the indicated times postinfection, RNA was extracted with Triozol (Invitrogen) and stored in water at −20°C. For passage of progeny virus, supernatant fluids were harvested at 48 h postinfection (hpi) and 300 μl was used to infect freshly grown C6/36 cells in elution buffer (1 mM EDTA, 0.5 M ammonium acetate), and ethanol precipitated. Cerenkow counts were determined by scintillation counting in water.

**Oligonucleotides used in this study.**

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<th>Oligonucleotide*</th>
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*The positive and negative symbols in the oligonucleotide names indicate the polarity of the nucleic acids to which the oligonucleotides anneal. Boldface nucleotides represent mutations. Polarity of the oligonucleotide relative to the positive-strand DI RNA. The positive and negative symbols in the oligonucleotide names indicate the polarity of the nucleic acids to which the oligonucleotides anneal. Boldface nucleotides represent mutations.

†For probe binding to positive-strand sequence, the numbers correspond to complementary positive-strand sequence.
RESULTS

SLV mapping within the nsp1 cistron immediately upstream of SLV is a required cis-acting signal for DI RNA replication. A 30-nt SL, SLVI, mapping at nt 101 to 130 within the 5′-terminal partial nsp1 cistron (genomic nt 311 to 340) (Fig. 1A and 2A) was recently identified as a cis-acting replication element for BCov DI RNA (12). Although its cis- replication feature remains to be tested in the context of the full-length viral genome, this SL along with the frame-shifting pseudoknot at the ORF 1a/1ab junction (9) are, to our knowledge, the only higher-order cis-replication elements known within CoV ORF 1 (12). Just upstream of SLVI is a structure-mapped 72-nt SL, SLV at nt 29 to 100 within the nsp1 cistron (genomic nt 239 to 310) (Fig. 2A) (12), whose function as a cis-acting higher-order structure has not yet been tested. To examine its potential cis-acting role in BCov DI RNA replication, synonymous base substitutions designed to disrupt base pairings in a helical region and compensatory mutations designed to restore base pairings were tested. Silent helix-disrupting substitutions were feasible in only the downstream side of the bottom-most stem, for which changes U306C and U309C were made to form mutant pRt (where Rt and Lt refer to the right and left sides of the stems, respectively), and transcripts were tested for DI RNA replication. Following transfection into BCov-infected cells, pRt DI RNA accumulation as determined by a visible band in Northern analysis was undetectable at 48 hpi and was present only at low levels in VP1 and VP2 compared to wt DI RNA levels (Fig. 2B). Sequence analysis of progeny RNA showed only wt revertants in VP2, indicating that the mutant had replicated poorly, if at all, and revertants with wt sequence, likely arising through recombination with the 5′ end of the helper virus genome, were selected (data not shown). However, when base pairings were restored by the compensatory mutations A240G and A243G in mutant pLt, replication as evidenced by accumulation of pLtRT DI RNA in Northern blots was restored to nearly wt levels in VP1 and VP2 (Fig. 2B), and mutations on both sides of the stem were retained in VP2 as revealed by sequence analysis (data not shown). A240G and A243G alone in mutant pLt were not predicted to disrupt base pairings in the stem, and, consistent with this structure, their presence alone did not block DI RNA replication, as evidenced by DI RNA accumulation in Northern blots (Fig. 2B) and sequence analysis. These results together indicate that optimal replication of DI RNA requires base pairing in the lowest helical region of SLV. Furthermore, when three silent point mutations were made in the upper loops of SLV, namely A264C, U273C, and G276A (Fig. 2A), to form the mutant named pLoops, the overall Mfold-predicted structure of SLV was only slightly changed (data not shown), and progeny DI RNA from pLoops, although slow in accumulating, retained the parental mutations in VP2. However, when the mutations in pLoops were combined with those in pRt to form pRt/Loops, there was additional structure disruption as predicted by Mfold, and accumulation was further impaired over pRt (Fig. 2B). That is, accumulation of pRt/Loops was not visible by Northern analysis at 48 hpi or in VP1 or VP2 (Fig. 2B) even though small amounts of wt progeny and progeny with reversions only at 306 and 309 were found by sequencing reverse transcription-PCR products (data not shown). These results
indicate that the overall higher-order structure of SLV, in addition to integrity in the lower stem, contributes to optimal BCoV DI RNA replication.

Translation of the partial 1a ORF has been shown to be a cis requirement for DI RNA replication (12, 14). To rule out the possibility that the observed loss of replication for SLV mutants was a result of unforeseen blocked translation arising from altered secondary structure (33), capped transcripts of wt and two selected mutants that did not replicate were translated in vitro in rabbit reticulocyte lysate, and the products were compared. Proteins translated in the presence of [35S]methionine from equal amounts of RNA (Fig. 2C) were evaluated by SDS-PAGE, and the results showed that wt, pRt, and pRt/Loops translated at comparable levels (Fig. 2C). Thus, the observed decreases in RNA accumulation can be attributed to defects in RNA synthesis and not defects in translation.

Unidentified cellular proteins with molecular masses of ~60 and 100 kDa, but not nsp1, bind cis-acting SLV and SLVI. To test our hypothesis that nsp1 binds intra-nsp1 cistronic cis-replication elements SLV and SLVI, two approaches were taken. In the first, evidence of viral protein binding to the 32P-labeled SLV-SLVI probe was sought by gel shift experiments with lysates of infected cells. In this analysis, three retarded proteinase K-sensitive complexes were found that were...
SLI-SLII and SLIV, to moderately bind the 3′-proximal octamer-associated bulged stem-loop (SLII) and SLIV, which nearly represents SLIII proper. Figure 4A (lanes 5 to 7) shows a complete inhibition of 50% of the lower form and all of the upper form of the probe fully shifting positions with 2.5 μM nsp1, indicating a $K_d$ (dissociation constant) of ~2.5 μM for nsp1 binding to SLIII-Long. Figure 4A (lanes 5 to 7) shows a complete inhibition of SLIII-Long shifting with 5, 10, and 15 μg of unlabeled probe, representing 2,500, 5,000, and 7,500 molar excess over labeled probe, respectively, whereas no inhibition was seen with 5, 10, and 15 μg of tRNA. These results together show specific binding of nsp1 to SLIII-Long.

**nsp1 binds SLIII and its flanking sequences with specificity and micromolar affinity.** Since nsp1 caused a quantitative shift in the SLIII-Long probe, we sought to establish both its specificity and affinity of binding to SLIII-Long, as shown in Fig. 1, and SLIII-short (SLIII with flanking sequences of only 10 nt upstream and 10 nt downstream), which nearly represents SLIII proper. Figure 4A, lane 1, shows that the SLIII-Long probe migrates as upper and lower bands, probably representing two folded forms of the probe. Lane 2 shows approximately 50% of the lower form and all of the upper form of the probe fully shifting positions with 2.5 μM nsp1, indicating a $K_d$ (dissociation constant) of ~2.5 μM for nsp1 binding to SLIII-Long. Figure 4A (lanes 5 to 7) shows a complete inhibition of SLIII-Long shifting with 5, 10, and 15 μg of unlabeled probe, representing 2,500, 5,000, and 7,500 molar excess over labeled probe, respectively, whereas no inhibition was seen with 5, 10, and 15 μg of tRNA. These results together show specific binding of nsp1 to SLIII-Long.

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The nsp1 cistron positioned for sgmRNA expression from DI RNA leads to inhibition of DI RNA accumulation but to only slight transient inhibition of viral RNA. To determine whether higher than normal levels of nsp1 expression have an effect on BCoV replication, as might be expected of a regulatory protein, pDrep-ISnsp1 was constructed for testing; for this construct the BCoV nsp1 cistron was cloned into the BCoV DI RNA vector pDrepIS12.7 at a site known to express subgenomic RNA in 1.5-fold molar excess over viral genome when the intergenic transcription signaling sequence for the mRNA 7 (mRNA for N synthesis) is used (Fig. 5A) (75). Overexpression of a viral protein from a DI RNA would avoid a noncoronaviral expression system, and the expressed protein would presumably be available to the viral replication machinery within its membranous compartment (55, 67). When BCoV-infected cells were transfected with T7 RNA polymerase-generated DI RNA from pDrep12.7 (wt) (a DI RNA expressing a 116-aa fusion protein from the transcription start site of viral sgmRNA 5 [47, 75]) or pDrep-ΔISnsp1 (which is an nsp1 cistron-containing DI RNA construct missing the 18-nt IS transcription start signal) and evaluated by Northern analysis with a probe detecting the downstream reporter sequence (HSVgD), DI RNA progeny for each was observed through VP2 (Fig. 5B). These results indicate little or no inhibition of DI RNA replication. However, from infected cells transfected with pDrep-ISnsp1 RNA, no increase in DI RNA accumulation over input RNA was detected at 24 h posttransfection or beyond (Fig. 5B and C). Nor were sgmRNAs with the nsp1 cistron identified by Northern analysis at these times posttransfection (data not shown) although they may have been present at earlier times posttransfection. In addition, no new bands were found that might have suggested recombination events between the DI RNAs and viral genome (data not shown). Therefore, direct evidence of nsp1 sgmRNA expression, as was shown for sgmRNA expression from pDrep12.7v(75), was not obtained. A blockage in the accumulation of DI RNA was not likely an effect of the nsp1 coding sequence within the DI RNA since the same sequence is present in pDrep-ΔISnsp1, which underwent replication. Interestingly, inhibition of DI RNA accumulation was observed not only with apparent expression of full-length nsp1 from a sgmRNA but also with expression of the C-terminal region of nsp1 from pDrep-ISnsp195-246 (genomic nt 493 to 948; nsp1 aa 95 to 246), representing a part of the genome whose homologous region in MHV can be deleted without loss of viral viability (Fig. 5B and C) (10). Less but still significant inhibition was seen with the N-terminal region of nsp1 from pDrep-ISnsp11-95 (genomic nt 211 to 495; nsp1 aa 1 to 95), a sequence whose homolog in the MHV nsp1 cistron contains a 30-nt sequence (nt 255 to 369) required for MHV genome replication (10, 21).

In addition, reduction in viral genome and sgmRNA levels with nsp1-expressing DI RNA was only slight and short-lived compared with expression levels in pDrep12.7-transfected cells (Fig. 5D to F). That is, a twofold reduction in viral sgmRNAs 6 and 7, the earliest appearing helper virus RNA species, was observed at 24 h posttransfection (Fig. 5E and F), but after this time wt levels were reached. No inhibition of viral genome or sgmRNAs was observed with expression of the C- and N-terminal regions of nsp1 (Fig. 5D to F).

We interpret these results together to mean that inhibitory levels of nsp1 were expressed from sgmRNA, although direct evidence for this expression remains to be shown, and that translation or replication of the DI RNA was more sensitive to the effects of nsp1 than that of viral genome or sgmRNAs. It is
FIG. 5. Detrimental effects of nsp1 expression from subgenomic mRNA in DI RNA. (A) Map of DI RNA constructs for expressing the full-length nsp1, the N-terminal 95 aa (N-term; aa 1 to 95), and the C-terminal 152 aa (C-term; aa 95 to 246) of nsp1. The IS used to induce sgmRNA transcripts for nsp1 expression is the 18-nt sequence upstream of the BCoV N mRNA (sgmRNA 7) (see text). (B) Northern analysis of in vivo generated transcripts of wt and truncated nsp1 constructs shown in panel A as identified by a probe specific for DI RNA (HSVgD reporter). Results from control constructs are also shown. ΔISnsp1 represents a construct identical to pDrep-ISnsp1 except that the 18-nt IS is deleted. RNA, a 1-ng sample of input RNA. (C) Quantitative summary of PhosphorImager data collected from data in panel B. (D) Northern analysis of viral RNA probed with a 3′ end-specific probe [3′UTR(+)]. sgmRNAs 6 and 7 are identified. (E and F) Quantitative summary of phosphor imaging data shown in panel D for sgmRNA 6 and sgmRNA 7, respectively. Asterisks identify the most inhibited quantities of sgmRNA 6 and sgmRNA 7 observed with nsp1 expression from DI RNA. Uninf, uninfected; inf, infected.
possible that as a transacting regulatory protein, nsp1 remains more localized in a DI-replicating compartment and does not freely diffuse to the replication-transcription complex of the viral genome.

**DISCUSSION**

Here, we test the hypothesis that nsp1 (p28) of BCoV, the first replicase protein to be synthesized and proteolytically released, is an RNA-binding protein that binds the cis-acting SLVI replication element within the nsp1 cistron. Binding of nsp1 to this structure would identify a potential means for autoregulation of gene expression. The rationale for this hypothesis is stated in the introduction. Although in this study we found no evidence of nsp1 binding to the intracistronic SLVI (12) as the hypothesis predicted, or to a newly identified SLV cis-acting replication signal immediately upstream of SLVI within the nsp1 cistron (this study), binding was found at other sites within the 5′ and 3′ UTRs that might function to regulate translation or replication. Notably, binding to a long form of cis-acting SLIII in the 5′ UTR with ~2.5 μM affinity was found.

To our knowledge, this is the first report of an RNA-binding property for CoV nsp1. While we tested binding here to only specific terminal regions of genomic RNA, the presence of multiple binding sites suggests possible broad-spectrum binding activity with other sites in the viral genome and also suggests relevance to the variety of cellular responses to CoV infection noted in several recent reports (16, 30, 45, 70, 79). Binding of nsp1 to cellular RNAs, for example, might induce pathogenic cellular responses. To date, however, only protein-protein interactions have been evaluated in these processes.

With regard to direct involvement of nsp1 in virus replication, several possibilities can be envisioned that will need testing. (i) nsp1 bound to SLIII (or SLs III and IV) might repress translation of ORF 1. The moderately high affinity of nsp1 for the long form of SLIII in the 5′ UTR and perhaps the lesser but still specific affinity to SLIV are consistent with results of an earlier study showing a 12-fold repression of translation in BCoV-infected cells for a reporter mRNA carrying the 210-nt genomic 5′ UTR but not one carrying the 77-nt sgmRNA 5′ UTR that experienced repression but not in the 77-nt sgmRNA 5′ UTR that experienced no repression (54). The molecules tested in these experiments had identical 5′ UTRs and perhaps the lesser 3′ UTRs (54). If the observed repression is a function of a viral protein as postulated (54), then nsp1 becomes a candidate for this function. It is intriguing that SLIII contains some portion of a short upstream ORF found in virtually all CoV genomes (50). Although the function of the upstream ORF has yet to be found, conceivably it or its translated product plays a role in regulating translation or RNA replication, and nsp1 by binding SLIII may influence these processes. Based on precedents in other well-studied positive-strand RNA viruses, an inhibition of translation might facilitate negative-strand synthesis in a couple of ways. (a) nsp1 binding might block ribosomal transit and enable initiation of negative-strand synthesis. (b) nsp1 might interact with SLs I to IV and also the 3′ end of other 3′ end-binding components, to form a circular molecule that functions to inhibit translation and enable initiation of negative-strand synthesis as has been shown for picornaviruses (5, 22). (ii) nsp1 might interact with a membrane-anchoring protein to bring the genome into the membrane-protected replication compartments, where the viral replication complex assembles and presumably functions in the absence (mostly) of the cellular translation machinery (55, 67). This mechanism, too, has precedence in nodaviruses and several plant viruses (68). (iii) nsp1 by binding directly to the very 3′ end of the genome might take part in replication complex formation and initiation of negative-strand synthesis. (iv) nsp1 through its affinity for SLIII in the 5′ UTR may play a role in RNA-dependent RNA polymerase template-switching events that direct leader formation at the 3′ end of sgmRNA-length templates for sgmRNA synthesis (74, 75). (v) Finally, nsp1 might function in the initiation of nascent positive strands from the 3′ end of the negative-strand antigenome. For this, a systematic study has not been made, but we have learned that SLIII does specifically, although weakly, bind the negative-strand counterpart to SLIII-Long and SLIII-Short (data not shown), suggesting a possible role for nsp1 in positive-strand synthesis.

At this time we have no experimental evidence to explain

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**FIG. 6.** Aligned amino acid sequences for nsp1 in BCoV (p28), MHV (p28), and SARS-CoV (p20). The arrowhead identifies the separation site of BCoV nsp1 into the N-terminal and C-terminal parts studied in the experiments shown in Fig. 5. The potential zinc finger-like RNA-binding domain in BCoV and MHV (nt 34 to 75) is identified. Also highlighted (gray shading) in all three viruses is a highly similar 8-aa site of BCoV nsp1 into the N-terminal and C-terminal parts studied in the experiments shown in Fig. 5. The potential zinc finger-like RNA-binding domain in BCoV and MHV (nt 34 to 75) is identified by highlighting. Also highlighted (gray shading) in all three viruses is a highly similar 8-aa site.
how expression of nsp1 from a DI RNA affects the accumulation of DI RNA more than that of helper virus genome and sgmRNAs, but it is possible that the DI RNA is simply more sensitive to nsp1 function or that it is in a different membrane compartment than the viral genome and sgmRNAs and therefore experiences a higher concentration of nsp1. The fact that both the N-terminal and C-terminal regions affect accumulation suggests that there is more than one functional domain within nsp1 involved in regulation. Nevertheless, a nearly complete repression of DI RNA accumulation and a twofold repression of viral sgRNA abundance, even if short-lived, is suggestive evidence that nsp1 has an effect on viral mRNA expression.

Both the identity and function of the ~60- and 100-kDa cellular proteins that bind SLV-SLV1 remain to be determined. Curiously, six cellular proteins with molecular masses of 25 to 58 kDa but no viral proteins were found to bind SLIV in the 5′ UTR (51). The arrangement of three cis-acting elements in close proximity suggests that they might function together as a larger structure. Although a relationship between cellular proteins binding SLV-SLV1 or SLIV-SLV1 and translation inhibition has not yet been shown, it is possible that the cellular proteins play a role in targeting the viral genome to the membranes making up the replication compartment (67).

Where is the RNA binding domain in nsp1? Bioinformatics analysis of the BCoV p28 amino acid sequence predicts no obvious RNA binding domain but does reveal a potential Zn finger with similarity to RNA binding motifs (13, 26). This domain (aa 34 to 75), common to both BCoV and MHV, is highlighted in Fig. 6. It has also been noted that an 8-amino-acid C-proximal domain of unknown function, also highlighted in Fig. 6, is conserved among all group 2 CoVs including the SARS-CoV, and the positively charged surface of this domain could be an RNA binding site (1). The functional RNA binding domain in nsp1, however, remains to be experimentally determined.

We conclude that, together, the results indicate that nsp1 is an RNA-binding protein that may function as a regulator of viral translation or replication but not through the binding of 5′-proximal cis-acting SLV and SLVI within its coding region. How and where nsp1 functions as an RNA-binding protein, and how SLV and SLVI function as cis- regulatory structures are questions that remain to be answered.

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