cis-acting RNA signaling elements that function directly in the replication of positive-strand viral genomes have been often first identified in helper virus-dependent defective or defective interfering (DI) RNAs (18, 26). One advantage in using DI RNAs for this identification is that cis-signaling functions for replication can be evaluated independently from other functions for the same structure. Such functions might include encoding a trans-acting factor or regulating the synthesis of a trans-acting factor (e.g., regulation of translation to produce a replicase). With the use of a 2.2-kb DI RNA of the bovine coronavirus (BCoV), a molecule composed of only the two ends of the genome fused between an internal site within the 738-nucleotide (nt) 5'-most coding region (the nsp1, or p28, coding region) and a site just 4 nt upstream of the 3'-most open reading frame (ORF) (the N gene). The joined ends of the viral genome in the DI RNA create a single continuous 1,635-nt ORF, 288 nt of which come from the 738-nt nsp1 coding region. Here, we have analyzed features of the 5'-terminal 288-nt portion of the nsp1 coding region within the continuous ORF that are required for DI RNA replication. We observed that (i) the 5'-terminal 186 nt of the nsp1 coding region are necessary and sufficient for DI RNA replication, (ii) two Mfold-predicted stem-loops within the 186-nt sequence, named SLV (nt 239 to 310) and SLVI (nt 311 to 340), are supported by RNase structure probing and by nucleotide covariation among closely related group 2 coronaviruses, and (iii) SLVI is a required higher-order structure for DI RNA replication based on mutation analyses. The function of SLV has not been evaluated. We conclude that SLV within the BCoV nsp1 coding region is a higher-order cis-replication element for DI RNA and postulate that it functions similarly in the viral genome.

Three cis-replication elements have been identified in the 289-nucleotide (nt) BCoV 3' UTR: (i) a bulged stem-loop beginning just downstream from the stop codon of the N gene (20, 21) and an adjacent pseudoknot (45), which possibly together act as a single higher-order molecular switch (14). The bulged stem-loop was first identified in the MHV genome (24). (ii) SLIII (nt 97 to 116) and an adjacent helical region (H. Y. Wu and D. A. Brian, unpublished). This structure was first identified in an MHV DI RNA (47). (iii) A 3'-terminal poly(A) tail (42; S. D. Senanayake and D. A. Brian, unpublished). Interestingly, to date only cellular proteins have been shown to interact with the 3' UTR structures, and of these, only the 3'-terminal octamer-associated bulged stem-loop region (32, 47, 48) and the poly(A) tail (42) have been shown to be targets. The cis-acting functions of the 3' UTR elements may be common among the group 2 coronaviruses, since the BCoV DI RNA can replicate with the helper function of several group 2 coronaviruses (46), and the entire 3' UTR of the BCoV and of human severe acute respiratory syndrome-associated coronavirus (SARS-CoV) can functionally replace the 3' UTR in the MHV genome (16, 21).
is predicted by both the Tinoco and Zuker algorithms, is supported by RNase structure probing, and appears to have a homolog in coronavirus groups 1 to 3 (34). In essentially all coronaviruses examined, a translation initiation codon for a short intra-5′ UTR open reading frame (ORF) of unknown function is associated with SLIII (34). Base covariations in the helical stem indicate that SLIII is phylogenetically conserved among group 2 coronaviruses (34 and data not shown). (iii) SLIV (nt 186 to 215) is a cis-acting phylogenetically conserved structure in group 2 coronaviruses with the exception of SARS-CoV (35). The structure of SLIV in SARS-CoV appears similar to those predicted for group 1 coronaviruses (35).

During initial studies to determine the structural requirements for BCoV DI RNA replication, it was learned that translation of the continuous ORF within the DI RNA was necessary for replication (7). Beyond this, it has not been determined how the partial nsp1 coding sequence functions in DI RNA replication. Interestingly, an analogous region of the nsp1 coding sequence is found in all naturally occurring and in one synthetically created coronavirus DI RNAs described to date (reviewed in reference 3). In addition, in the context of one synthetically created coronavirus DI RNA, the nsp1 coding sequence is found in all naturally occurring and in the translation of the continuous ORF within the DI RNA was examined. It was learned that translation of the DI RNA was necessary for DI RNA replication on the basis of mutation analyses. We conclude that SLVII within the BCoV nsp1 coding region acts as a higher-order cis-replication element for DI RNA and postulate that it functions similarly in the viral genome.

MATERIALS AND METHODS

Cells, virus, and DI RNA. A DI RNA-free stock of the Mebus strain of BCoV (genome sequence; GenBank accession no. U00735) at 4.5 × 106 PFU/ml was used as a helper virus as described previously (7, 8). The human rectal tumor cell line HRT-18 (43) was used in all experiments. pDrep1 is a pGEM3Zic(-) (Promega)-based plasmid containing the cDNA clone of a naturally occurring 2.2-kb proximal 288 nt of the nsp1 coding region in the DI RNA ORF. The sequence was not determined, but it was speculated to be the nsp1 coding sequence, but not of the 3′-proximal region, prevents genome replication as determined through reverse genetics analyses (6). The mechanistic contribution of this sequence was not determined, but it was speculated to be perhaps a function of the protein product, an RNA structure, or both (6).

In this study, we have examined the function of the 5′-proximal 288 nt of the nsp1 coding region in the DI RNA ORF that provides replication competence to the BCoV DI RNA. We observed that (i) the 5′-terminal 186 nt of the nsp1 coding region are necessary and sufficient for DI RNA replication, (ii) within the 186 nt sequence are found two Mfold-predicted stem-loops, named SLV (nt 239 to 310) and SLVI (nt 311 to 340), that are supported by RNase structure probing and by nucleotide covariations that maintain the helical stems among the closely related group 2 coronaviruses, and (iii) SLVI is a required higher-order structure for DI RNA replication on the basis of mutation analyses. The function of SLV has not been evaluated. We conclude that SLVI within the BCoV nsp1 coding region acts as a higher-order cis-replication element for DI RNA and postulate that it functions similarly in the viral genome.

Northern assay for DI RNA replication. The Northern assay for detecting reporter-containing DI RNAs was performed as described previously (8, 33). Briefly, 5 μg of MluI-linearized plasmid DNA was blunt ended with mung bean nuclease and transcribed with 40 U of T7 RNA polymerase (Promega) in a 100-μl reaction mixture to produce uncapped DI RNA. The reaction mix was treated with 5 μl of RNase-free DNase (Promega), and RNA was chromatographed through a BioSpin 6 column (Bio-Rad) and quantitated spectrophotometrically. 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 1 μg of RNA using Lipofectin (Bethesda Research Laboratories). At the indicated times postinfection, RNA (approximately 10 μg per plate) was extracted by the Nonidet P-40-protease K method (8) and stored as an ethanol precipitate. For passage of progeny virus, supernatant fluids were harvested at 48 h postinfection (hpi), and 500 μl was used to infect freshly confluent cells (~8 × 106) in a 35-mm dish from which RNA was extracted at 48 hpi. For electrophoretic separation of RNA in a formaldehyde-agarose gel, 2.5 μg per lane was used. Approximately 1 ng of transcript, identified as RNA in the Northern blot figures, was loaded per lane when used as a marker. RNA was transferred to Nytran membrane by vacuum blotting, and the UV-irradiated blots were probed with oligonucleotide TGEV(+) that had been 5′-end labeled with [32P] to specific activities of 1 × 106 to 4 × 107 cpm/pmol. Protein blots were exposed to Kodak XAR-5 film for 1 to 7 days at ~80°C for imaging.

Reverse transcription-PCR and sequence analysis of mutant DI RNA replicons. With RNA extracted from cells at 24 h postinfection with VPI virus collected from mutant transfections, reverse transcription was done using DI at ~20°C. For RNA treatments, 40 μg of RNA was heat denatured at 65°C for 3 min and renatured by slow cooling (0.5 h) to 35°C in a 400-μl reaction volume containing 30 mM Tris HCl (pH 7.5)–20 mM MgCl2–300 mM KCl, and aliquots containing 2 μg of sample RNA and 10 μg of yeast tRNA were incubated in 100 μl of the same buffer and 1.0, 0.1, 0.05, 0.01, or 0.001 U of RNase CV1 (Phar- macia) or 5.0, 1.0, or 0.1 U of RNase T2 (GIBCO), as noted below in the legend to Fig. 3. RNAse digestion was carried out at 25°C for 15 min and terminated by the addition of 150 μl of 0.5 M sodium acetate, after which the RNA was extracted with phenol-chloroform and ethanol precipitated. Digested RNA prep- arations were used in primer extension reactions with 5′-end-labeled plus-strand- binding primer 1 (BCoV332-352 for mapping SLV) or primer 2 (BCoV339-400 for mapping SLVI) (Table 1). Undigested RNA was used with the same primers to identify natural reverse transcription stops and in dideoxynucleotidyl sequenc- ing reactions to mark nucleotide positions. Products were analyzed on a DNA sequencing gel of 6% polyacrylamide.

Construction of mutant DI RNAs. Plasmids from which mutant DI RNAs were transcribed in vitro were modifications of pDrep1 (8). Modifications of pDrep1 were made by overlap PCR mutation amplification (19, 38, 45) using pDrep1 DNA template and the oligonucleotide primers described in Table 1. Restriction endonu- clease sites were used for final construction of the mutant plasmids as shown in Fig. 1A. Mutated regions were confirmed by sequencing.

To make p77Drep, the 210-nt 5′ UTR in pDrep1 was shortened by overlap mutagenesis wherein the products from primers PGEM3Z297(+), BCoV477–74(+) (DI-112-232(+)), and oligo 2(+) were extended, and the mutated 1,158-nt NdeI fragment from the extended product (the upstream NdeI site is within the vector) was used to replace the analogous fragment in pDrep1. To make p210Nrep, pDrep2 (8) was digested with BglII, and the resulting 754-nt BglIII fragment was used to replace the analogous fragment in pDrep1. 3′-end deletions of the 288-nt partial nsp1 coding region within pDrep1 were made by overlap PCR mutagenesis wherein primers Nstarr(+) and oligo 2(−) were used to make the 3′-proximal overlapping fragment and primers PGEM3Z2477(−) and the indicated mutant primers (Table 1) were used to make the 5′-proximal overlapping fragments of differing lengths. The mutated Hpal-XbaI fragments from the final extended PCR products were used to replace the analogous fragment in pDrep1. The name of each deletion mutant corresponds to the primer used with the same name.

pLefVI was made by PCR overlap mutagenesis wherein primers PGEM3Z2477(-), Leftvi(+), Leftvi(−), oligo 2(−) were used to make an overlap PCR product from which the mutated 1,158-nt NdeI fragment was used to replace the analogous fragment in pDrep1. pRightVI was made in the same way except for the use of primers Rightvi(+) and Rightvi(−), respectively.

In vitro translation. For in vitro translation, ~2 μg of transcript was transcribed in a 50-μl reaction mix containing 35 μl of rabbit reticulocyte lysate (Promega) and 20 μCi of [35S]methionine (~800 Ci/mmol; MP Biochemicals) as recom- mended by the manufacturer. Radiolabeled proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in gels of 12% polyacrylamide (Tris-HCl Ready gel; Bio-Rad) by the method of Laemmli (25), and dried gels were scanned for imaging.

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FIG. 1. Location of cis-replication elements within the partial nsp1 coding region in BCoV DI RNA. A. Structure of the naturally occurring BCoV DI RNA relative to the full-length BCoV genome. The DI RNA is composed of the fused ends of the viral genome as described in the text. The 65-nt leader is illustrated by a filled rectangle. The partial nsp1 sequence in parentheses represents the 5′-proximal 288 nt of the 738-nt nsp1 coding region. The cloned, modified BCoV DI RNA is under control of the T7 RNA polymerase promoter, carries a 30-nt in-frame reporter used for Northern probing, and is named pDrep1. Restriction endonuclease sites used for further engineering as described in this report are depicted. The cloned, modified BCoV N mRNA (mRNA 7) is under control of the same promoter as pDrep1, carries the same reporter, and is named pNrep2. The difference in the RNA transcripts from the two plasmids both linearized at the MluI site is a continuous sequence of 421 nt as depicted. At the right are shown the results of Northern analyses depicting the accumulation of pDrep1 RNA at 48 and 96 h posttransfection and at 24 hpi following first virus passage (VP1), and the absence of accumulation of the pNrep2 RNA. RNA, 1 ng of transcript used for transfection.

B. Structures of modified pDrep1 that carry the 5′ UTR of mRNA 7, named p77Drep, and the 5′ UTR of the genome but without the 288-nt region of nsp1, named p210Nrep. At the right are Northern analyses depicting the accumulation of wt DI RNA and the absence of accumulation for RNAs from p77Drep and p210Nrep. RNA, 1 ng of transcript used for transfection.

C. Deletion analysis of the 288-nt partial nsp1 coding region in BCoV DI RNA. 3′-terminal deletions of the partial nsp1 coding region were made as indicated, and RNAs from the mutants were tested for accumulation. The results of Northern analyses at the right illustrate that DI RNAs carrying the first 186 nt of nsp1 coding region accumulated, whereas those with less than 186 nt did not. RNA, 1 ng of transcript used for transfection.
RNA-specific primer TGEV(+), PCR was done using primers TGEV(+) and leader(-), and the PCR product was sequenced directly.

RESULTS

The 288-nt partial nsp1 coding region provides a required function for BCoV DI RNA replication. Previous studies had demonstrated that a T7 RNA polymerase-generated sgRNA 7 (mRNA for the N protein) of BCoV containing a short reporter sequence does not replicate when transfected into BCoV-infected cells, whereas the DI RNA containing the same reporter sequence does (8). The difference between the two molecules is a continuous stretch of 421 nt that is made up of some of the genomic 5′ UTR sequence and all of the 288-nt partial nsp1 region (Fig. 1A). To test whether the genomic 5′ UTR and the partial nsp1 coding region independently fulfill requirements for replication of the DI RNA, constructs were made that had the 210-nt genomic 5′ UTR, forming p210Nrep. From this plasmid, a DI RNA-like molecule was made that had the 210-nt genomic 5′ UTR and all the N-coding domain and reporter sequence but was missing the entire 5′-proximal 288 nt of the nsp1 coding domain. Figure 1B illustrates that a transcript of p210Nrep also failed to replicate in helper virus-infected cells, thus demonstrating a requirement for all or part of the 288-nt partial nsp1 coding region in the DI RNA for replication.

cis-acting signals for DI RNA replication map within the 5′-terminal 186 nt of the partial nsp1 coding region in the DI RNA, a region containing SLV and SLVI. To characterize the cis-acting features within the 288-nt partial nsp1 coding region, conserved sequences and higher-order structures among eight group 2 coronaviruses previously studied (BCoV-Mebus, human coronavirus [HCoV]-OC43, HCoV-4408, porcine hemagglutinating encephalomyocarditis [HEV]-TN11, equine coronavirus [ECoV]-NC99, classed as BCoV-like, and mouse hepatitis coronavirus [MHV-A59, MHV-2, and MHV-JHM], classed as MHV-like [46]), were identified and treated as potential cis-acting elements. For this, sequences were aligned (46) and the Mfold program of Zuker applied to each. Four predicted hairpin stem-loops showing nucleotide variations in the helical stems among the eight viruses were identified and named SLV through SLVI. In the reporter-containing mRNA 7 construct (pNrep2) (8) was precisely replaced with the 210-nt genomic 5′ UTR, forming p210Nrep. From this plasmid, a DI RNA-like molecule was made that had the 210-nt genomic 5′ UTR and all the N-coding domain and reporter sequence but was missing the entire 5′-proximal 288 nt of the nsp1 coding domain. Figure 1B illustrates that a transcript of p210Nrep also failed to replicate in helper virus-infected cells, thus demonstrating a requirement for all or part of the 288-nt partial nsp1 coding region in the DI RNA for replication.

TABLE 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Polarity</th>
<th>Sequence (5′-3′)</th>
<th>Binding region nt in pDrep</th>
</tr>
</thead>
<tbody>
<tr>
<td>1aA217-498 (+)</td>
<td></td>
<td>GAGTAAAAGACATG/CACATTGGTG</td>
<td>207–216/499–511</td>
</tr>
<tr>
<td>1aA238-498 (+)</td>
<td></td>
<td>GAGTAAAAGACATG/GAGACCTATTTTG</td>
<td>225–237/499–511</td>
</tr>
<tr>
<td>1aA283-498 (+)</td>
<td></td>
<td>GAGTAAAAGACATG/TCTGCGTCTTC</td>
<td>274–282/499–511</td>
</tr>
<tr>
<td>1aA319-498 (+)</td>
<td></td>
<td>GAGTAAAAGACATG/CACCTCTGAAC</td>
<td>308–318/499–511</td>
</tr>
<tr>
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<td>GAGTAAAAGACATG/TGTTCACGCC</td>
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</tr>
<tr>
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<td>GAGTAAAAGACATG/ACAACTCCACCATC</td>
<td>384–396/499–511</td>
</tr>
<tr>
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<td>398–408/499–511</td>
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<tr>
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<td>412–423/499–511</td>
</tr>
<tr>
<td>1aA442-498 (+)</td>
<td></td>
<td>GAGTAAAAGACATG/TATTAAGCTAG</td>
<td>431–441/499–511</td>
</tr>
<tr>
<td>1aA454-498 (+)</td>
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<td>442–453/499–511</td>
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<tr>
<td>1aA478-498 (+)</td>
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<tr>
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<td>CCAGGGTACCAGGCTGGACCTGGTG</td>
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<td>GGCGGACAATCCACCATACCATG</td>
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<tr>
<td>DI211-232 (−)</td>
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<td>CGTTATTTGTGATCTTGACATCTTTAAAGTGTAG</td>
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<tr>
<td>Leader 7-32 (−)</td>
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<td>GAGCGATTTTGCCGTCGGTCATCCGCC</td>
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<td>+</td>
<td>GTAGTTCAAGATGCGATATAGTGTCGAAGTCGATTTG</td>
<td>305–329</td>
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<tr>
<td>LeftVI (+)</td>
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<td>RightVI (−)</td>
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</tr>
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<td>+</td>
<td>GCGCAGTAGTGTGAGAGATCATAATAC</td>
<td>319–343</td>
</tr>
<tr>
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<td></td>
<td>CATGCGACACCACCTCTTGGCAACCCAGA</td>
<td>1098–1123</td>
</tr>
</tbody>
</table>

a The positive and negative symbols in oligonucleotide names indicate the polarity of the nucleic acid to which the oligonucleotide anneals.

b Polarity of the oligonucleotide relative to the positive-strand DI RNA.

c For probe binding to negative-strand sequence, the numbers given correspond to complementary positive-strand sequence.

d Binds in the pGEM3zf(−) vector upstream of the DI RNA transcription start site.

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kCal/mol, respectively (Fig. 2). Identical bases aligning among the eight coronaviruses are shown in Fig. 2, and those that differ are identified. Although the SARS-CoV has been classified as a group 2 coronavirus (41), structural homologues of stem-loops V to VIII in SARS-CoV were not apparent by these analyses (data not shown).

To determine whether the entire nsp1 portion present in the DI RNA molecule is required for DI RNA replication, a set of 3′-terminal in-frame deletions of this region in pDrep1 was made by overlap PCR mutagenesis, and T7 RNA polymerase-generated transcripts of each mutant were tested for replication in helper virus-infected cells. Figure 1C summarizes the data showing that deletion mutants 1a/H9004 478-498 through 1a/H9004 397-498 replicated, as evidenced by accumulation of DI RNA after VP1 and VP2, whereas 1a/H9004 358-498 through 1a/H9004 217-498 did not. A low level of DI RNA accumulation prior to virus passage posttransfection (for example, the undetectable amounts for mutant p454-498 at 24 h and 48 h posttransfection in Fig. 1C) may reflect structural influences on replication, but we noted that amounts of accumulation at these times did vary widely, even for wt DI RNA (e.g., compare Fig. 1 and 4, below). Thus, for DI RNA replication, the 3′-terminal 102 nt of the 288-nt partial nsp1 coding region, which contains most of SLVIII, are not needed. It was therefore concluded that the SLV- to SLVII-containing 5′-proximal 186-nt region contains one or more cis-acting elements required for DI RNA replication. Of these, SLV and SLVI were chosen for further analysis since they possessed relatively low free energies and demonstrated sequence conservation in the stems.

**R**Nase structure maps are consistent with predicted stem-loops V and VI. To determine whether stem-loops V and VI as predicted by Mfold are supported by enzyme structure probing, synthetic transcripts of the first 288 nt of the nsp1 coding region were probed by methods used previously to analyze stem-loops I through IV and the 3′-proximal pseudoknot (9, 34, 35, 45). For this, pDrep1 was linearized at the XbaI site and T7 RNA polymerase-generated transcripts were digested with various concentrations of RNase specific for single-stranded regions (RNase T2 [nonspecific with a preference for A]) and helical regions (cobra venom RNase V1). The positions of cleavage sites in the RNA (summarized in Fig. 3A) were determined by analyzing primer extension products in parallel with a dideoxy sequencing ladder generated from untreated RNA (Fig. 3B and C). Primer extension products of uncut RNA were also examined to identify natural reverse transcription stops. The results showed that for SLV via extension of primer 1 (Fig. 3B), strong single-strand hits were present at nt 227 to 230, a predicted single-stranded region for (A); the positions of cleavage sites in the RNA (summarized in Fig. 3A) were determined by analyzing primer extension products in parallel with a dideoxy sequencing ladder generated from untreated RNA (Fig. 3B and C). Primer extension products of uncut RNA were also examined to identify natural reverse transcription stops. The results showed that for SLV via extension of primer 1 (Fig. 3B), strong single-strand hits were present at nt 227 to 230, a predicted single-stranded region between SLIV and SLV, at nt 268 and 269, sites within and adjacent to a predicted 2-nt bulge, and at nt 273 to 276, sites within the predicted 4-nt terminal loop. In addition, weak single-strand hits were observed at nt 267, a site within a 2-nt bulge, at nt 272, a site adjacent to the terminal loop, at nt 282, a site not identical bases aligning among the eight coronaviruses are shown in Fig. 2, and those that differ are identified. Although the SARS-CoV has been classified as a group 2 coronavirus (41), structural homologues of stem-loops V to VIII in SARS-CoV were not apparent by these analyses (data not shown).

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adjacent to an internal loop, and at nt 298 and 299, sites within a predicted internal loop. Strong double-strand hits were present at nt 222 to 226, a previously unmapped region that appears to extend the length of SLIV described earlier (35) (to be analyzed further elsewhere). Strong double-strand hits were also present at nt 242 and 243, a predicted double-stranded region, at nt 263 and 264, adjacent to and within an internal loop, and at nt 277, adjacent to the terminal loop. In addition, weak double-strand hits were observed within stems at nt 255, 262, 265, 269, 270, 278, 288, and 310 and within loop regions at nt 258 and 268. Double-strand hits in predicted loop regions may be explained by the fact that CV1 on occasion digests within single-stranded regions, adopting an approximately helical conformation (27).

The mapping results for SLVI as observed via extension of primer 2 (Fig. 3C) show strong single-strand hits at nt 321 to 325, which represent 5 of the 10 nt in the predicted terminal loop. Strong double-strand hits were present at nt 317 to 319, a region predicted to be in the upper stem.

Thus, RNase probing with T2 and CV1 RNases overall
yielded results consistent with the stem-loop V and VI structures predicted by Mfold.

Mutation analyses show that SLVI plays a functional role in DI RNA replication. Between SLV and SLVI, SLVI was chosen for testing a cis-replication function since it is relatively small and lends itself to translationally silent stem-disrupting nucleotide changes. It also demonstrated a high level of nucleotide sequence conservation in its upper stem compared with other group 2 coronaviruses (46) (Fig. 2).

To test the replication function of SLVI, base substitutions were made in the third (wobble) nucleotide positions of four base-paired codons in the upper stem that were predicted to disrupt helical structure but preserve wild-type amino acid sequence (Fig. 4A). In construct pLeftVI, substitutions 315G→A and 318G→C were made, and following transfection of transcripts into virus-infected cells no DI RNA accumulation was observed at 24 and 48 h posttransfection, nor was replication observed at 24 h following the first passage of virus (Fig. 4B, LVI). Likewise, when mutations 333C→G and 336C→T were made to form pRightVI, no replication was observed (Fig. 4B, RV1). However, when compensatory restoration of stem-loop VI was made by combining the mutations in pLeftVI and pRightVI, replicating ability was restored as evidenced by the accumulation of DI RNA at 24 and 48 h posttransfection and by replication following the first virus passage (Fig. 4B, L/RVI). Sequence analysis of cDNA clones made from VP1 RNA of the replicating L/RVI double mutant demonstrated that all four mutations were retained (data not shown).

Since the mutations made in SLVI resulted in codons used in the BCoV genome (57% of the time for GAA [Glu], 8% of the time for GUC [Val], 26% of the time [19 times total] for UCG [Ser], and 48% of the time for ACU [Thr]), it was not anticipated that blocks in DI RNA replication would result from failed translation of the mutated DI RNA ORFs. To establish that translation was not blocked by the mutations, transcripts of the mutant DI RNAs were translated in rabbit reticulocyte lysate, and the products were examined. The results shown in Fig. 4C demonstrate that wt and all three mutant forms of DI RNA are translated to generate the fused DI RNA ORF product of approximately 62 kDa. The apparent larger size for this protein is due to its phosphorylation (data not shown).

From these combined results, it was concluded that stem-loop VI, as a higher-order structure, is a required cis-acting element for BCoV DI RNA replication.

Whether SLV functions similarly remains to be determined.

**DISCUSSION**

In the current study, we report the existence of a higher-order RNA structure identified as SLVI within the nsp1 coding region of the coronaviral genome that appears to act directly in cis for DI RNA replication. SLVI maps within a part of the nsp1 coding region found in all naturally occurring coronavirus DI RNAs (3, 4, 8) and in one synthetic MHV-A59 DI RNA (30) described to date, suggesting that a structure analogous to SLVI may be required for the replication of these DI RNAs as well. In at least one other instance, an apparently directly acting higher-order cis-replication structure has been found to map within the coding region of a coronavirus DI RNA. In that case, the structure, a 55-nt stem-loop derived from the nsp 3 ORF, exists in a subset of multipartite DI RNAs generated from MHV-JHM (36). Other cis-acting RNA structures have been mapped within the coding region of the coronavirus genome but appear to function indirectly in genome replication (29). These include the ribosome frameshifting pseudoknot located just downstream of the ORF 1a/1b junction (5, 13), the (usually) intergenic core sequence (also called the transcription regulatory sequence) associated with RdRp template switching during formation of sgRNA templates (50), and a genome packaging signal that maps within ORF 2b (11). A cis-replication element has been described within a coding region of an arterivirus (2, 44), a virus group that along with coronaviruses, toroviruses, and roniviruses is a member of the Nidovirus group. This cis-acting structure is in the 3′-proximal region of the arterivirus genome, however, and to date appears not to have a homolog in the coronavirus genome.

Whether SLVI is the only directly acting cis RNA replication element within the 5′-terminal region of ORF 1a remains to be determined. SLV is a complex stem-loop that shows considerable structural conservation among the group 2 coronaviruses but has yet to be examined for a cis-replication function. Curiously, like SLVI, the terminal loop of SLV appears to be the most variable part of its structure. The proximity of SLV and SLVI suggests they may be components of a single larger cis-acting structure.

It also remains to be determined what role the protein moiety of the partial nsp1 product plays as a cis-acting feature, if
any. It was initially thought that translation of the partial ORF 1a sequence in the BCoV DI RNA is also required for replication (7). Replacing the AUG start codon for the partial nsp1 ORF in DI RNA with a UAG stop codon resulted in no DI RNA replication, even when the N portion of the DI RNA ORF remained open (7). The notion that translation of the partial nsp1 ORF is required for DI RNA replication has been supported by two additional experiments in which the amino acid composition within short regions of the partial nsp1 product was changed and tested. In the first mutant, a nuclease deletion at position 223 (causing a −1 frameshift) and a nuclease insertion at position 304 (causing a +1 frameshift) caused amino acids 5 through 31 to change from 5NKGYGLELHWAPEFPMFDASEELDNP31 in the wt DI RNA to 5TNTVSNYGLQNFHCLRTQRRSWITL31 in the mutant. In the second mutant, a nuclease deletion at position 270 (causing a −1 frameshift) and a nuclease insertion at position 304 (causing a +1 frameshift) caused amino acids 20 through 31 to change from 20MFEDAEEKLDNP31 in the wt DI RNA to 20CLRTRRWSITL31 in the mutant. Both of these mutations map within the region of SLV. Neither mutant replicated (data not shown). Thus, it could be that a feature of the translated partial nsp1 product fulfills a cis requirement for replication, but it cannot be ruled out at this time that the nuclease insertions and deletions altered a required cis-acting RNA structure.

To date, nsp1 has been the most characterized in MHV and SARS-CoV. In MHV, nsp1 is a 28-kDa protein, also called p28, the first proteolytically cleaved product of the polyprotein synthesized from ORF 1a (12). When p28 is expressed in the absence of the other viral proteins, it has profound effects on cell metabolism, including the induction of cell cycle arrest at G1/S (10). Interestingly, by using reverse genetics it was shown that the C-terminal region of p28 is not absolutely required for virus replication but that deletion of the N-terminal region is lethal for the virus (6). Expression of the SARS-CoV nsp1 protein alone in cells promoted host mRNA degradation (23).

The identification of cis-act replication elements in the large coronavirus genome remains a technical challenge but is now approachable with the use of reverse genetics systems (reviewed in reference 29). An important caveat when using DI RNAs for this identification is that such elements might be idiosyncratic for the DI RNA genome. One example of this is the coronavirus common octamer (GGAGAGGC) in the 3′ UTR which functions as a cis-act replication element in DI RNA but not in the full-length genome (15). On the basis of the work presented here, we suggest that the presence of a cis-act element for genome replication mapping within the 5′-proximal region of nsp1 may be one reason the 5′-proximal region of nsp1 cannot be deleted from the intact MHV genome (6). The presence of a cis-act element within the partial nsp1 coding region along with SLIII and -IV in the BCoV 5′ UTR could also explain why it is that the sgRNAs produced by this virus, although they are found in double-stranded RNA-synthesizing transcriptive intermediates (1, 37), are not replicating molecules as once postulated (17, 39, 40).

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