Minus-Strand Copies of Replicating Coronavirus mRNAs Contain Antileaders

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The 5' leader sequence on mRNAs of the porcine transmissible gastroenteritis coronavirus was determined and found to be 90 nucleotides in length. An oligodeoxynucleotide with a sequence from within the leader was used as a probe in Northern analysis on RNA from infected cells, and an antileader (a minus-strand copy of the leader sequence) was shown to be present on all mRNA minus-strand species. RNase protection analysis showed the antileader to be approximately the same length as the leader. The kinetics of antileader appearance was the same as that for the appearance of minus-strand RNA species. This, along with a demonstration that viral mRNAs become packaged, gives further support to the idea that coronavirus mRNAs can undergo replication via subgenomic mRNA-length replicative intermediates, and that input mRNAs from infecting virions may serve as initial templates for their own replication. In this sense, then, coronaviruses behave in part like RNA viruses with segmented genomes.

We have recently demonstrated that coronavirus mRNAs undergo replication via mRNA-length replicative intermediates (21). Although first demonstrated for the porcine transmissible gastroenteritis coronavirus (TGEV), the phenomenon has now been demonstrated as well for the mouse hepatitis virus (MHV) (19; unpublished data), and the bovine coronavirus (6). mRNA replication is therefore probably a general feature of coronavirus reproduction.

We postulate that mRNAs replicate by using the same mechanism(s) as the genome. A critical assumption in our postulate is that mRNAs possess a 3'-terminal sequence identical to that on the genome and that anti-mRNAs possess a 3'-terminal sequence identical to that on the antigenome (at least similar enough that they are recognized by the polymerase[s] for synthesis of minus and plus strands). That is, for TGEV we assume that there is (i) a 3'-terminal sequence of 1,518 nucleotides (nt) [exclusive of the poly(A) tail of unknown length] on each mRNA as well as on the genome and that some part of the sequence must serve as a promoter for minus-strand synthesis (this sequence includes the very 3'-most open reading frame for the 9.1-kDa hydrophobic protein and a 3' noncoding sequence [8]), and (ii) a 3' antileader sequence on the minus-strand copy of each mRNA that would contain the promoter sequence for plus-strand synthesis. Point (i) has been established by sequencing cDNA clones of the TGEV genome (8-10, 17) and by analogy with other coronaviruses for which mRNAs have been sequenced (see reference 22 for a review). In this report we establish point (ii) by determining the TGEV leader sequence and showing by Northern hybridization analysis with sequence-specific oligodeoxynucleotide probes that an antileader sequence exists on each mRNA minus-strand species. The size of the antileader was approximated by RNase protection analysis. We further demonstrate by showing the existence of intravirion mRNAs that packaged mRNAs are potential templates for their own replication.

MATERIALS AND METHODS

Cells and virus. Growth of the Purdue strain of TGEV on swine testicle (ST) cells and preparation of virus stocks have been previously described (3, 8, 21).

Determination of the TGEV leader sequence. To establish the TGEV leader sequence, an 18-base oligodeoxynucleotide (5' CGTTGTCCCTGGTTGGCC 3') complementary to bases 3 through 20 of the open reading frame of the nucleocapsid protein gene (8) was synthesized, 5' end labeled with polynucleotide kinase and [γ -³²P]ATP (>3,000 Ci/mmol; ICN Pharmaceuticals), and used as primer for an extension reaction (8) on cytoplasmic RNA harvested 6 h postinfection (prepared as described earlier [21]). The extended primer was isolated by electrophoresis on a 12% polyacrylamide sequencing gel and sequenced by the method of Maxam and Gilbert (15). This is essentially the same method as that used to determine the leader sequence of MHV (11) and the avian infectious bronchitis virus (IBV) (4). To confirm the leader sequence, a 26-base oligodeoxynucleotide (5' CAGCATG GAGGAAGACGAGCATCTCG 3') complementary to bases -4 through 22 of the hydrophobic protein (HP) gene (8) was used to determine the sequence at the 5' end of the HP mRNA in the same way.

Identification by Northern RNA analyses of leader on mRNAs and antileader on mRNA minus strands and size determination of the antileader. To test for the presence of leader and antileader sequences by Northern analyses and to construct a leader-containing DNA clone, four oligodeoxynucleotides, designated L1, L2, L3, and L4, were synthesized (Fig. 1A). L1 (80 nt) and L2 (47 nt) were plus (genome) sense, and L3 (80 nt) and L4 (47 nt) were minus sense. All were designed to overlap and anneal in such a way as to reconstruct the entire (90-nt) leader along with the adjacent AACTAAAC intergenic consensus-containing sequence and to generate *EcoRI* and *SacI* sites at the 5' end (plus sense) and *AvaI* and *BamHI* sites at the 3' end.

To detect leader sequences on plus-strand mRNAs by Northern analysis, oligodeoxynucleotide L3 was 5' end labeled with ³²P as described above and used to probe RNA blots prepared from formaldehyde-agarose gels as previously described (21). To detect antileader on mRNA minus

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strands, oligodeoxynucleotide L1 was likewise end labeled and used.

To construct a full-length DNA clone of the leader with adjacent intergenic consensus sequence and flanking restriction endonuclease recognition sequences, oligodeoxynucleotides L1, L2, L3, and L4 in equimolar concentrations were annealed, ligated with T4 ligase, digested with *SacI* and *AvaI*, and directionally ligated into the *SacI* and *AvaI* sites of the multiple cloning region of pGEM-3Z vector (Promega Biotech). The resulting clone was called pTGEV-L.

To determine the size of the antileader sequence, plus-sense, full-length transcripts of the radiolabeled leader and adjacent AACTAAAC intergenic sequence were generated from the pTGEV-L construct (Fig. 1A) and used in RNase protection experiments on cytoplasmic RNA isolated from infected cells. To generate radiolabeled transcripts, pTGEV-L DNA was linearized with *BamHI*, which cuts on the template strand 9 bases downstream from the 3' end of the AACTAAAC consensus sequence and within the multiple cloning region of the vector, and incubated with T7 RNA polymerase (Promega Biotech) in the presence of [α - 32 P] CTP (>600 Ci/mmol; ICN Biochemicals). The resulting radiolabeled transcripts thus had the sequence 5' gggc gaaucgagcucuuu-[leader]-AACUAAACccgaggatc 3' and a total length of 127 nt (Fig. 1). The first four bases were derived from the transcription start site in the pGEM-3Z vector. Approximately 10^6 cpm (an estimated 2 μ g) of radiolabeled RNA transcript was incubated in 300 mM NaCl with 30 μ g of cytoplasmic RNA, extracted from either uninfected ST cells or from TGEV-infected cells harvested at 6 h postinfection, and digested with 2 μ g of RNase T₁ (Calbiochem) per ml and 40 μ g of RNase A (Sigma) per ml as described by Sambrook et al. (18). Cytoplasmic RNA was extracted as previously described (21) and digested with 50 μ g of RNase A per ml in 300 mM NaCl for 1 h at 37°C to remove the large excess of plus-strand species before reextraction and hybridization to radiolabeled, plus-strand transcripts. (This procedure is similar to one known to enhance identification of small numbers of picornavirus minus strands [16].) Protected RNA was identified on a denaturing DNA sequencing gel of 6% polyacrylamide beside a DNA ladder of known sequence.

Purification and treatment of virus for examination of intravirion RNA species. To prepare virus for examination of intravirion RNA species, freshly confluent ST cells were infected at a multiplicity of 10 PFU per cell and supernatant fluids were harvested at 16 h postinfection, when 10 to 25% of the cells were showing cytopathic effect. Supernatant fluids (900 ml, total) were gently drained from cells and clarified by centrifugation at $2,000 \times g$ for 20 min, and virus was sedimented onto a cushion of 60% (wt/wt) sucrose by centrifugation at $90,000 \times g$ for 1 h at 4°C. Virus was then isopycally sedimented in an 18-ml gradient of 60 to 20% (wt/wt) sucrose by centrifugation at $90,000 \times g$ for 4 h at 4°C and harvested from the top by using an Isco gradient fractionator. Sucrose solutions were made up in 10 mM Tris-maleate (pH 6.0)–1 mM EDTA–100 mM NaCl. Twenty 1-ml fractions were collected, the visible virus band (with a buoyant density of 1.19 to 1.20 g/cm³ as previously described [3]) was isolated, and virus was suspended into two equal portions (3.5 ml each). RNase A was added to one portion to a final concentration of 20 μ g/ml, and both portions were incubated for 90 min at 37°C; the virus was then pelleted, and RNA was extracted as previously described (21). RNA was stored at -20°C as a precipitate under 70% ethanol (8.5 ml total for each pellet); for electrophoresis, RNA from 90 μ l of

the precipitate suspension (prepared by vigorous vortex mixing) was used per lane.

Nucleotide sequence accession number. The GenBank accession number of the TGEV leader sequence given in Fig. 1A is M33310.

RESULTS

TGEV leader sequence is 90 nt long. The TGEV leader sequence is defined here as the sequence on the 5' end of the subgenomic mRNA molecules that does not have a colinear counterpart in the genome. It is the same definition that was used to describe the leader on IBV (4) and is not to be confused with the free leader of the type hypothesized to play a role in leader priming of transcription from genome-length minus-strand RNA (2, 11). If TGEV were to possess a free leader of this type, it would be expected to be longer than just the 5' unique sequence and to extend in the 3' direction to include the intergenic consensus sequence (2, 11). From the sequence of the extended oligodeoxynucleotide primer that bound within the N gene, the TGEV leader was found to diverge from genomic sequence at a point 15 nt upstream from the N initiation codon (8) and to extend 90 nt to its 5' terminus (Fig. 1A). The identity of the 5'-terminal base is not clear, but it appeared to be the first U of a 5-base poly(U) tract. It is not yet known whether TGEV mRNAs possess 5'-terminal methylated caps of the kind described for MHV (11). To confirm the leader sequence, an extended primer from within the HP gene (8) was also sequenced; the leader sequence was identical to that derived from the N primer, except that it diverged from the genome sequence 2 nt farther upstream, a point 14 nt upstream from the HP initiation codon. For both the N and HP mRNAs, the 90-nt leader is followed immediately by the 8-base sequence AACUAAAC, which contains the consensus intergenic sequence CUAAAC (8–10, 17). The 5'-terminal 98-nt sequences of the N and HP mRNAs are therefore identical (Fig. 1A).

Antileaders are found on minus-strand copies of subgenomic mRNAs. To test for the presence of leader sequence on all remaining mRNAs of TGEV (i.e., those for the M, 9.2K, 27.7K, 7.7K, S, and polymerase [genome] proteins), the 80-base oligodeoxynucleotide L3, which is complementary to bases 31 through 98 of the combined leader and intergenic consensus sequence (Fig. 1A), was 5' end labeled with 32 P and used in a Northern analysis on cytoplasmic RNA prepared at various times postinfection (Fig. 1B, lanes 1 through 10). The experimental protocol was the same as that used previously (21). Although a sequence complementary to the AACUAAAC intergenic consensus-containing sequence was part of the probe (and therefore would recognize the intergenic sequence on leaderless mRNAs), the washing conditions used (21) were too stringent to allow this sequence alone to be the cause of hybridization. Thus, any hybridization observed would have been the result of binding by a large portion of the leader sequence. From Fig. 1B, lanes 1 through 10, it is evident that all mRNA species were identified in the Northern analysis, indicating that all contain the leader sequence.

To test for the presence of antileader sequence on subgenomic minus-strand RNAs, the 80-base oligodeoxynucleotide L1, with the sequence of the 5'-most 63 bases of the leader (a region that excludes the consensus intergenic sequence; Fig. 1A), was 5' end labeled and analyzed in a Northern RNA blot identical to the one described above for detecting leader sequence. Although fewer molecules hy-

FIG. 1. (A) DNA version of TGEV leader sequence, with the adjacent AACTAAAC consensus-containing sequence and flanking restriction endonuclease recognition sequences. The 90-nt TGEV leader sequence (bases 18 through 107) and adjacent consensus-containing sequence (underlined) are shown in uppercase letters. Lowercase letters at the ends identify bases added to introduce restriction endonuclease sites in the synthetic construct. L1 and L2 identify sequences of oligodeoxynucleotides having these names. L3 and L4 identify sequences that are complementary to oligodeoxynucleotides L3 and L4, respectively. (B) Detection of leader on mRNAs and antileader on mRNA minus strands. A leader-detecting probe (L3) was used in Northern hybridization analysis of RNA from virion, uninfected cells, or infected cells at the times indicated (lanes 1 through 10). An antileader-detecting probe (L1) was likewise used as indicated (lanes 11 through 20). An unidentified antileader-containing species is marked by an asterisk. Lanes 11 through 20 were exposed to film five times longer than were lanes 1 through 10.

bridized (both probes were labeled to nearly the same specific activity of 3×10^6 cpm/pmol), each minus-strand species identified in our earlier study (20) was identified by the antileader-detecting probe (Fig. 1B, lanes 11 through 20). An antileader-containing species for which we have no explanation appeared between αn and αm (Fig. 1B). The lower number of probe molecules detecting the antileader as compared with those detecting the leader is apparently a reflection of the 10-fold-lower number of minus-strand molecules (21). The kinetics of appearance of both leader and antileader are similar to the kinetics of appearance of both plus- and minus-strand RNA species as measured in our earlier studies (21) by hybridization to sequence-specific oligodeoxynucleotide probes from within the HP gene.

TGEV antileader is approximately 90 nt long. To determine the size of the antileader sequence, a full-length, radiolabeled positive-sense leader transcript containing the leader, together with the flanking AACUAAAC consensus sequence and restriction endonuclease sequences, was used in RNase protection assays, and the size of the product was measured. The majority of full-length radiolabeled transcript was 127 nt and migrated on a 6% polyacrylamide denaturing sequencing

gel coincident with the DNA marker of 127 nt (Fig. 2, lane 1). The protected fragment should thus be the size of the antileader, together with its adjacent consensus sequence. When RNA from infected cells was hybridized and subjected to RNase treatment in high salt, six fragments ranging in size from 83 to 94 nt were observed; the most abundant were fragments 88 and 89 nt in length (Fig. 2, lane 6). None of these was present when RNA from uninfected cells was used (Fig. 2, lane 7). There are two possible explanations for these results. (i) The antileaders and adjacent GUUUAGUU sequences on the majority of anti-mRNAs are not complete complements of the leader and adjacent intergenic sequence on mRNAs and are in fact shorter by a few nucleotides. Such a set of molecules would represent a heterogeneous antileader population in which there is variation in only a few nucleotides. (ii) The antileader and adjacent GUUUAGUU sequence are fully present on mRNAs but are not fully protected under the hybridization conditions used. Of these possibilities, we favor the latter, since the phenomenon of hybrid breathing in terminal A+U-rich regions is well documented in RNase protection studies (1) and the conditions for this behavior exist here. It is possible, for example, that

FIG. 2. Size of the antileader sequence. The full-length radiolabeled leader and the adjacent consensus-containing sequence (plus sense) were hybridized to cytoplasmic RNA and subjected to RNase digestion under conditions (high salt) that would protect hybrids (see Materials and Methods). The resulting products were phenol extracted, ethanol precipitated, and electrophoresed on a DNA sequencing gel of 6% polyacrylamide. Lanes: 1, 127-base radiolabeled plus-sense, full-length transcript; 2, 3, 4, and 5, products from a DNA sequencing reaction for DNA of known sequence; 6, protected RNA from infected cells; 7, protected RNA from uninfected cells.

some portion of the 13-base A+U-rich region at the 5' end of the leader (a region containing only one G residue) would open and allow the single-strand-specific RNases to cleave internally. Cleavage at G by RNase T_1 would yield a fragment of 89 nt, and cleavage at the sixth U residue by RNase A would yield a fragment of 88 nt. Other cleavage products are also possible.

mRNAs are packaged into virions. These and earlier studies (21) have suggested that TGEV packages its mRNA species (lane 1 in Fig. 1B). To determine whether the mRNA species appearing with purified virions are packaged or are noninternalized species adhering to the virion surface, virions purified by isopycnic sedimentation in a sucrose gradient were digested with RNase A before virion pelleting and RNA extraction. RNA from RNase-treated virions was analyzed by Northern hybridization with a 26-nt oligonucleotide complementary to sequence within the HP gene as described earlier (21). RNA from RNase-treated virions contained all mRNA species (Fig. 3). The molar abundance of the subgenomic mRNA species, however, ranged from 0.07 to 0.2 that of the full-length genome as determined by measuring radioactivity in excised bands (21).

The existence of antileader on mRNA minus strands and the packaging of mRNAs are probably universal properties

FIG. 3. Detection of packaged TGEV subgenomic mRNAs. Northern analysis with a plus-strand-detecting probe was done as previously described (21) on RNA isolated from purified virus that had undergone incubation at 37°C only (lane 1) or incubation with RNase (lane 2) before virion pelleting and RNA extraction.

of coronaviruses, since we have also demonstrated them for the bovine coronavirus (unpublished data). Interestingly, for the bovine coronavirus, mRNAs for the N and M proteins are packaged more abundantly (in terms of number of molecules per virion) than is the genome (6).

DISCUSSION

This report is the first description of the 5' leader sequence on mRNAs of TGEV, a representative of a third major antigenic subgroup of coronaviruses. IBV and MHV represent the two other major antigenic subgroups (22). The leader of TGEV is distinctly longer, 90 nt as compared with 58 nt for IBV and 61 nt for MHV, if the leader is defined as the 5'-terminal sequence on subgenomic mRNAs with no colinear counterpart in the genome (Fig. 4). One striking comparative feature is that, although TGEV and MHV share nearly identical consensus sequences (CUAAAC) located on the 3' side of the leader, within the leader sequence itself there is abundantly more sequence homology between TGEV and IBV than between TGEV and MHV. Over the entire region of overlap among the three leaders (TGEV leader bases 28 through 73, 47 nt), there is 55% (26 of 47) sequence homology between TGEV and IBV, and 28% (13 of 47) homology between TGEV and MHV. Interestingly, a region of significant homology (47%) exists between TGEV and IBV over a sequence that is within the leader sequence of TGEV (TGEV leader bases 74 through 90) but within the genome sequence of IBV.

Two common notions concerning coronavirus replication require reexamination as a result of the demonstration that antileaders exist on minus-strand copies of replicating mRNAs. The first is that the full-length genome is sufficient for virus replication (summarized in the model of Fig. 5A). Although coronavirus RNA has been shown to be infectious (3, 13, 20, 24), neither single-hit kinetics of genome infectiv-

from mRNA replication. Splicing of plus-strand precursors, therefore, remains a viable hypothesis to explain the origin of mRNAs. (This model has been described in papers by Baric et al. [2] and Spaan et al. [23].) Because antileaders exist on subgenomic minus strands, it can be alternatively considered, as suggested by Sawicki and Sawicki (19), that splicing of a minus-strand genome-length precursor gives rise to subgenomic minus-strand templates.

Finally, the replication of mRNAs may explain how a large number of molecules of only a few of many possible mRNA subspecies are readily found in MHV-infected cells (14). Subspecies of a given mRNA were characterized that differed only in the number of UCUAA pentanucleotide repeats, or in some cases UAUA and UCCAA repeats, in the putative fusion region between the leader and body of the mRNA molecule. Subspecies of RNAs containing 1, 2, 3, or 4 UCUAA repeats were proposed to have arisen from an imprecise priming event. Since an imprecise event of this kind would be expected to give rise to a small number of a wide variety of possible molecules, rather than the other way around, a more likely explanation is needed. We suggest that mRNA replication is such a mechanism, since it would generate many faithful copies of a given mRNA subspecies, perhaps one that arose from an imprecise priming event.

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