CORONAVIRUS SUBGENOMIC REPLICONS AS A MECHANISM FOR mRNA AMPLIFICATION

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INTRODUCTION

Assuming promoter sequences for synthesizing antigenome lie within the 3' noncoding region on the coronavirus genome (approximately 300 bases), and the promoter for synthesizing genome lie within the antileader sequence (approximately 80 bases) on the minus strand, we asked why do mRNAs not replicate? Coronavirus mRNAs have been shown to possess 3' and 5' noncoding regions in common with the genome (Brown *et al.*, 1984; Brown *et al.*, 1986; Lai *et al.*, 1984; Shieh *et al.*, 1987). If mRNA replication occurs, then we would predict the existence of (1) subgenomic (mRNA-length) minus strands, (2) subgenomic (mRNA-length) double-stranded replicative forms, and (3) subgenome mRNA replication rates that exceed that of the genome. We present evidence that suggests these three conditions are fulfilled for TGEV, and we, therefore, conclude that TGEV mRNAs undergo replication. We propose that this is a mechanism for mRNA amplification, a mechanism not yet described for any other RNA virus family having a nonsegmented genome.

MATERIAL AND METHODS

The Purdue strain of TGEV was plaque purified from infectious genomic RNA and a stock of virus made from passage 5 as previously described (Brian *et al.*, 1980; Kapke *et al.*, 1988) was used. Swine testicle cells in roller bottles were infected with a multiplicity of 10 and RNA was extracted as previously described (Kapke *et al.*, 1988) except that 10mM vanadyl ribonucleoside complex was added to the lysis buffer. RNA (10 μ g per lane for cytoplasmic RNA and 20ng per lane for virion RNA) was electrophoresed in 1% agarose-formaldehyde gels essentially as described (Dennis and Brian, 1982) except that 20mM Mops buffer (pH7.0) was used. RNA was vacuum blotted onto Nytran membrane (Schleicher and Schuell) and bound by UV-crosslinking. Blots were hybridized with a synthetic oligodeoxynucleotide probe (probe 1; 5'-CAGCATGGAGGAAGACGAGCATCTCG-3') that is complementary to virus-sense RNA within the HP gene (Fig. 1), and that had been 5' end-labeled to a specific activity of 1.6 to 3.5x10⁶ cpm per pmole by the forward reaction using [γ -³²P] ATP (ICN). Probe 2 has a sequence that is complementary to probe 1 and it was likewise end-labeled. Membranes were prehybridized at 55°C for 2h in 5X SSC (1X SSC is 0.1%

Ficoll/0.1% polyvinyl pyrrolidone/0.1% bovine serum albumin/50 mM sodium phosphate, pH 7.0/1% SDS) that contained 100 μ g sheared salmon sperm DNA and 50 μ g tRNA per ml. Radiolabeled probes ($\approx 2 \times 10^7$ Cerenkov cpm per 120 cm^2 membrane) were denatured at 90°C for 5 min and added to the prehybridization solution, and hybridization was carried out at 55°C for 16h. The membrane was given three 10-min. washes in 2xSSC at 25°C and a fourth wash for 30 min at 55°C, air dried and exposed to Kodak XAR-5 film. Radioactive bands were excised for liquid scintillation counting in Scintiverse. The number of molecules of each mRNA species per cell was determined from the specific activity of the individual probe and from our measured yield of 10 μ g of RNA per 1.6×10^6 cells.

For identification of double-stranded replicative forms, intracellular RNA extracted at 6 hpi was used. 60 μ g of cytoplasmic RNA was dissolved in 40 μ l 10mM Tris-HCl, pH 7.2/300mM NaCl/10mM MgCl_2 /1mMEDTA containing 10 μ g RNase A (Sigma) per ml, and incubated at 37°C for 30 min. Digestion was terminated by adding SDS to a final concentration of 2% and RNA was extracted with phenol-chloroform, and precipitated. RNA was analyzed by electrophoresis and blot hybridization as described above.

RESULTS

We and others have established the nucleotide sequence of the 3'-terminal 8.5 kilobases of the TGEV genome and have identified seven open reading frames (Fig. 1; Jacobs *et al.*, 1987; Kapke and Brian, 1986; Kapke *et al.*, 1988; Kapke *et al.*, 1989; Laude *et al.*, 1987; Rasschaert and Laude, 1987; Tung, 1987). mRNA transcripts have been identified for each of these ORFs by metabolic labeling (Dennis and Brian, 1982; Jacobs *et al.*, 1986). To confirm that each transcript and genomic RNA can be identified by Northern blotting experiments, RNA blots were probed with a G+C-rich 26 mer complementary to a region within the HP gene (Fig. 1). Fig. 2 demonstrates that genomic RNA, and mRNAs for P, 7.7K, 27.7K, 9.2K, M, N and HP were each identifiable by this procedure, and yielded a pattern that is consistent with the 3' coterminal nested set structure of coronavirus mRNAs. Furthermore, by counting individual bands, an estimate of the number of molecules per cell for each species was made (Fig. 3A).

To determine whether subgenomic minus strands exist, probe 2 (which is complementary to probe 1) was used in Northern analyses (Fig. 2). A minus-strand counterpart (identified by α) for the genome and for each mRNA species was identified. Their numbers were likewise estimated (Fig

Fig. 1. Gene map for TGEV and site of the HP specific probes. The seven ORFs in the 3' end of the TGEV genome are drawn to scale. P=peplomer protein, M=matrix protein, N=nucleocapsid protein, HP=hydrophobic protein, and 7.7K, 27.7K, and 9.2K ORFs are potential nonstructural proteins. Corresponding mRNAs are respectively identified as p, m, n, hp, 7.7k, 27.7k, and 9.2k. Arrow identifies site from which the probe sequences came.

3B). Four observations suggested that probes designed to detect minus strands were not merely detecting abundant mRNA molecules nonspecifically. (1) Three of the eight minus-strand species (α hp, α 27.7k, and α 7.7k) did not migrate with the same mobility as did their presumed plus-strand counterparts. (2) A probe from within the N gene designed to detect minus strand, did so, and also detected the larger minus-strand species, but not α hp (data not shown). (3) The maximal abundance of minus-strand species occurred at 4 hpi., whereas that of plus strands occurred at 6 h.p.i. (Fig. 3). (4) Probes used to detect minus-strand species did not identify virion genomic RNA (Fig. 2, lane 20). We do not yet know the detailed structure of the molecules identified as high mol. wt. RNA and those identified by asterisks in Fig. 2.

To determine whether subgenomic double-strand replicative forms exist cytoplasmic RNA from infected cells at 6 h.p.i. was treated with pancreatic RNase A in 0.3M NaCl, and RNase resistant forms were denatured, electrophoresed, blotted and hybridized to probe 1 or probe 2 (Fig. 4A). Both plus and minus strands corresponding in size to mRNA species were found indicating that subgenomic replicative forms were present. Furthermore, within double-stranded forms, very few full-length (i.e., mRNA-length) plus strands were found, but full-length minus strand RNAs were abundant. We interpret this to mean that the replicative forms represented replicative intermediate structures on which a single minus-strand was serving as template for multiple plus strands (Fig. 4B). Plus strand molecules would have been degraded to protected species too small to be resolved in our electrophoretic system.

Both plus- and minus-strand subgenomic RNA species were, in general, synthesized at a rate inversely related to their length (Fig. 3, A and B). This can be seen by noting slopes throughout the first 6h for plus-strand

Fig. 2. Subgenomic plus- and minus-strand RNA species identified by hybridization. RNA from purified virions or from cytoplasm of uninfected or infected cells (hours postinfection are shown above the lanes) was electrophoresed, blotted, and hybridized with radiolabeled probe 1 to detect plus-strand RNA species (lanes 1-10) or with probe 2 to detect minus-strand RNA species (lanes 11-20). α indicates antisense (or minus strand) polarity; three dots at the top of each lane identify the well.

RNA synthesis, and throughout the first 4h for minus-strand RNA synthesis.

DISCUSSION

To our knowledge, this is the first demonstration of subgenomic minus-strand RNA for coronaviruses. The experimental approach used here may demonstrate subgenomic minus strands for other coronaviruses. We have shown them to exist for the bovine enteric coronavirus (M. Hofmann, P. Sethna, and D. Brian, unpublished data), for example, a close relative of the mouse hepatitis virus for which subgenomic minus strands were not demonstrable by other experimental methods (Lai *et al.*, 1982).

The phenomenon of subgenomic mRNA replication does not rule out a mechanism like that of leader-primed transcription to explain the origin of subgenomic mRNAs. A mechanism such as leader-primed transcription must exist to explain the origin of mRNAs from a single, infectious coronavirus genome (Lai, 1986; Spaan *et al.*, 1983). Once made, however, the individual subgenomic mRNA could serve as template for its replication. Conceivably, some mRNA species could enter the cells as part of the virion since they are known to be packaged (P. Sethna and D. Brian, unpublished data; Fig. 5).

Two biological consequences can be predicted from the replication of subgenomic mRNAs. First, replication may serve as an efficient mechanism to rapidly amplify mRNAs required for structural protein synthesis, thus freeing the full-length genome for genome replication and polymerase synthesis. Second, replicating mRNAs would most probably compete with genome for limiting factors required in RNA replication (such as the RNA polymerase) and in this way behave as genomes of defective interfering viruses. This may be a mechanism by which coronaviruses readily establish persistent infections in cell culture.

Fig. 3. Kinetics of plus strand (A) and minus-strand (B) RNA synthesis. Radiolabeled bands in Fig. 2 were excised and counted. Copy numbers were determined as described in Materials and Methods.

Fig. 4. (A) RNase-resistant species in replicative forms. Cytoplasmic RNA obtained from cells 6 hr postinfection was digested with RNase A in 0.3 M NaCl, electrophoresed in a denaturing gel, transferred and hybridized with probes from the HP gene sequence. Lane 1, 10 μ g of untreated RNA, and lane 2, 30 μ g of RNase-treated RNA, were hybridized with probe 1. Lane 3, 10 μ g of untreated RNA, and lane 4, 30 μ g of RNase-treated RNA, were hybridized with probe 2. Lanes 5 and 6 are extended exposures of lanes 1 and 2. (B) Models showing the relationship between the replicative intermediate (RI) and resulting double-stranded replicative form (RF) following RNase digestion for a replicative intermediate of 2.5 kilobases. Sites to which probes 1 (on the plus strand) and 2 (on the minus strand) would bind are depicted by a filled circle and an open circle, respectively.

Fig. 5. Two schemes by which mRNAs potentially become available for replication. (1) mRNAs are derived by a mechanism such as leader-primed transcription. (2) mRNAs are carried into the cell by the infecting virion.

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