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Characterization of leader-related small RNAs in coronavirus-infected cells: Further evidence for leader-primed mechanism of transcription

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Summary

Mouse hepatitis virus (MHV), a murine coronavirus, replicates in the cytoplasm and synthesizes 7 viral mRNAs containing an identical stretch of leader RNA sequences at the 5′-end of each RNA. The leader-coding sequences at the 5′-end of genomic RNA are at least 72 nucleotides in length and are joined to the viral mRNAs by a unique mechanism. Utilizing a leader-specific cDNA probe, we have detected several free leader RNA species ranging from 70 to 82 nucleotides in length. The predominant leader RNA was approximately 75 nucleotides. In addition, larger distinct leader-containing RNAs were also detected ranging from 130 to 250 nucleotides in length. The 70–82-nucleotide leader-related RNAs were present in both the cytosol and membrane fractions of infected cells. They were also detected only in the small RNA fractions but not associated with the replicative-intermediate RNA. These data suggest that the leader RNAs were associated with the membrane-bound transcription complex but at least part of them were dissociated from the RNA template. We have also identified a temperature-sensitive mutant, which synthesizes only leader RNA but not mRNAs at nonpermissive temperature, indicating that leader RNA synthesis is distinct from the transcription of mRNAs. These data support the leader-primed mechanism for coronavirus transcription and suggest that one or more free leader RNAs are used as primers of mRNA synthesis.

Mouse hepatitis virus, transcription, small leader related RNAs, coronavirus, temperature-sensitive mutant

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Introduction

Mouse hepatitis virus (MHV), the prototype of Coronaviridae, contains a single-stranded and plus-sensed RNA of 5.4×10^6 daltons (Lai and Stohlman, 1978). The 60S genome is enclosed within a helical nucleocapsid constructed from multiple copies of capsid protein, pp60 (Sturman, 1977; Stohlman et al., 1983). During maturation, the nucleocapsid buds into endoplasmic reticulum, forming an intact virion containing an envelope and two virus-specific glycoproteins, gp 23 and gp 180/90 (Sturman, 1977; Wege et al., 1979; Siddell et al., 1981).

Murine coronaviruses are normally associated with hepatitis in vivo and infect a variety of established cell lines in vitro (Wege et al., 1982). These viruses replicate in the cytoplasm, independently of nuclear involvement (Brayton et al., 1981; Wilhelmsen et al., 1981). Upon entry, the viral genome is translated into an 'early' polymerase which directs the synthesis of full-length (−)-sensed RNA (Lai et al., 1982b; Brayton et al., 1984). This enzymatic activity is replaced later in infection by two separate 'late' polymerase activities which synthesize either genomic or mRNAs, respectively. These enzymatic activities are associated with cellular membranes (Brayton et al., 1982, 1984).

Seven virus-specific mRNAs ranging from 0.6 to 5.4×10^6 daltons are detected on polysomes of virus-infected cells (Lai et al., 1981, 1982a; Leibowitz et al., 1981; Spaan et al., 1981). These RNAs are arranged in the form of a nested set from the 3′-end of the genome such that the sequences of each smaller RNA are contained within the 3′-sequences of the next larger RNA species (Lai et al., 1981). In addition to this unique genetic arrangement, it has recently been demonstrated that each mRNA and genomic RNA contain an identical stretch of approximately 72 nucleotides at their 5′-ends (Lai et al., 1983, 1984; Spaan et al., 1983). The presence of a leader RNA common to all viral mRNAs indicates that they are synthesized by joining two noncontiguous RNA segments, since the leader sequences are encoded only at the 5′-end of the genomic RNA (Lai et al., 1983, 1984; Spaan et al., 1983). These data suggest that a unique mechanism of RNA synthesis occurs during MHV infection. This mechanism differs from conventional eukaryotic RNA splicing since UV transcriptional mapping studies indicate that these mRNAs are not derived from cleavage of large precursor RNAs (Jacobs et al., 1981). The study of replicative-intermediate RNA further suggests that the leader RNA is joined to the mRNAs during transcription, but not post-transcriptionally (Baric et al., 1983). We have also shown that the double-stranded replicative form (RF) is of genome length, suggesting that the joining mechanism does not involve 'looping out' of intervening sequences in the (−)-stranded RNA template since such a mechanism would have generated subgenomic RFs (Baric et al., 1983). Thus, these data are compatible with a model in which the coronavirus leader RNA is synthesized independently and then sequestered to prime transcription of the body sequences of each mRNA by binding specific initiation sites on the (−)-strand template (Baric et al., 1983). This unique mechanism is supported by sequence data from mRNAs 6 and 7 (Armstrong et al., 1984; Spaan et al., 1983; Skinner and Siddell, 1981), which demonstrate the presence of sequence complementarity between the 3′-portion of the leader RNA and

intergenic 'start' sites on the (−) strand. Presently, the exact length of the leader RNA is not known but must consist of 57 nucleotides from the 5′-end of the genome plus at most 15 nucleotides which are complementary to the splice junctions on the (−)-stranded RNA (Spaan et al., 1983).

In this communication, we demonstrate the synthesis and characterization of small leader-containing RNAs in MHV-infected cells. Moreover, we have isolated a temperature-sensitive mutant which accumulates a leader RNA of approximately 72 nucleotides in length at the nonpermissive temperature. These data support the 'leader-primed' mechanism for coronavirus transcription (Baric et al., 1983) and demonstrate another mode of RNA synthesis in eukaryotic cells. The implications of these findings will be discussed.

Materials and Methods

Virus and cells

The A59 strain of MHV was propagated in DBT or L2 cells as previously described (Lai et al., 1981). Temperature-sensitive mutants of A59 were isolated on L2 cells following mutagenesis with 5-fluorouracil or 5-azacytidine. The detailed protocol on mutant isolation and description of phenotypes of these mutants will be published elsewhere. Experiments utilizing these mutants were performed at permissive (32°C) and nonpermissive (39°C) temperatures.

Preparation of MHV intracellular RNA

The intracellular RNA from A59-infected cells was isolated between 5 and 7 h post-infection in the presence of 2 μg/ml actinomycin D. The RNA was extracted with phenol/SDS as described in earlier publications (Lai et al., 1981).

Synthesis of leader-specific cDNA

The synthetic oligodeoxyribonucleotide (15-mer) (5′-AGTTTAGATTAGATT-3′) was prepared by Dr. Bruce Kaplan, Department of Molecular Genetics, City of Hope Medical Center, Duarte, Calif. The 15-mer was 5′-end-labeled with [γ-^{32}P]ATP by polynucleotide kinase and hybridized to total intracellular RNA from the MHV-infected cells as previously described (Lai et al., 1984). After ethanol precipitation and several washings, leader-specific cDNA was synthesized with reverse transcriptase in a buffer containing 1 mM each of dATP, dGTP, TTP and dCTP, 10 mM $MgCl_2$, 120 mM KCl and 30 mM mercaptoethanol at 42°C for 1 h. The RNA template was removed by alkaline treatment, and the full-length cDNA product (72 nucleotides long) was isolated by molecular sieve chromatography on a Sephadex G-50 column.

S_1 mapping

The leader-specific cDNA was synthesized as described above, except that [α-^{32}P]dCTP was used instead of unlabeled dCTP in the reaction. The products were separated on 12% polyacrylamide gels containing 6 M urea, located by autoradiogra-

phy, and the ^{32}P-labeled 72-nucleotide leader cDNA eluted in DNA extraction buffer (0.5 M ammonium acetate, 0.01 M Mg acetate, 0.001 M EDTA and 0.1% SDS) for 12 h at 65°C. Following ethanol precipitation, the cDNA was hybridized to the 60S genomic RNA isolated from the purified virus in 80% formamide, 0.4 M NaCl, 0.01 M Na phosphate (pH 7.0), and 0.01 M Pipes-HCl (pH 6.8) at 37°C for 5 h. The hybrid was washed extensively and digested with 1000 units of S_1 nuclease in 0.2 M NaCl, 0.05 M Na acetate (pH 4.5), 0.0018 M zinc sulfate and 0.5% glycerol for 1 h at 37°C. The reaction mixture was adjusted to 0.01 M EDTA and extracted with phenol/chloroform prior to electrophoresis on 12% polyacrylamide gels.

Subcellular fractionation

Infected cells, harvested between 5 and 6 h post-infection, were swollen in 0.5 × RSB buffer (1 × RSB: 0.01 M Tris-HCl, pH 7.4/0.01 M NaCl/0.0015 M $MgCl_2$) for 30 min at 4°C. The cells were gently Dounce-homogenized and centrifuged at 1000 × *g* for 5 min. The nuclear pellet was suspended in the same buffer, Dounce-homogenized and repelleted to increase the purity of this preparation. Following disruption of the nucleus with 0.5% SDS, nucleic acids were extracted as described. The supernatant, containing membrane and cytosol, was centrifuged at 36 000 rpm (100 000 × *g*) in an SW 41 rotor for 90 min. Under these conditions, membranes were not detected in the S-100 fraction by electron microscopy and over 95% of the activities of membrane-associated enzyme markers, e.g. (Na^+-K^+)-ATPase and 5′-nucleotidase were contained within the P-100 fractions. The cytosol fraction (S-100) was adjusted to 0.5% SDS and extracted by the phenol/chloroform method (Lai et al., 1981). The membrane fraction (P-100) was repelleted as described, suspended in 0.5 × RSB containing 0.5% SDS and extracted with phenol/chloroform.

Polyacrylamide gel electrophoresis and electroblotting protocols

Equivalent amounts (25 or 50 μg/well) of RNA were separated by electrophoresis on 12 or 20% polyacrylamide gels in TBE buffer (0.1 M Tris-borate, pH 8.3/0.002 M EDTA) containing 6 M urea. The RNA was electrophoresed at 1000 V until the bromophenol blue marker had migrated 15–20 cm from the origin. Under these conditions, intracellular RNAs smaller than 250 nucleotides in length enter the gel matrix while the viral mRNAs are excluded. Urea was removed from the gel matrix by several washings of cold TAE buffer (0.04 M Tris-base/0.02 M Na acetate/0.01 M EDTA, pH 7.4) and the RNA electroblotted to Zeta probe paper (Bio-Rad) in cold TAE. Electroblotting was performed in a circulating chamber at 340 mA for 10–15 h. This protocol efficiently transfers RNA molecules smaller than 250 nucleotides while larger nucleic acids are retained in the gel (Bittner et al., 1980). Following transfer, the paper was gently washed in TAE and baked at 80°C for 2 h prior to hybridization with ^{32}P-labeled probes according to published procedures (Thomas, 1980).

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Genomic RNA   .....CAUUGAAUCUAAUCUAAACUUUAAG.....
    mRNA #7   .....GUUUAAAUCUAAUCUAAACUUUAAGGAUGUC.....

    mRNA #6   .....GUUUAAAUCUAAUCUAAACUAUAUG.....
 Leader RNA   .....GUUUAAAUCUAAUCUAAACU.....
                        60          70
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Fig. 1. Comparison of the junction sequences encoded on genomic RNA, mRNAs 6 and 7 and the leader RNA. The sequences shown are the leader–body junction regions of mRNAs 6 and 7 and the 3′-end sequences of leader RNA (Armstrong et al., 1984; Spaan et al., 1983; Lai et al., 1984). The genomic sequences are from the region which corresponds to the initiation sites for mRNA 7 (Spaan et al., 1983). The sequences underlined represent the common sequences and the possible leader–body junction sites.

Results

Size analysis of leader-coding sequences on the genomic RNA

It has previously been determined that the length of the leader RNA is approximately 72 nucleotides (Armstrong et al., 1984; Lai et al., 1984). This estimate was obtained by primer extension of a synthetic oligodeoxyribonucleotide (5′-AGTTTAGATTAGATT-3′) (15-mer), which is complementary to a portion of the splice junction sites of MHV mRNAs 6 and 7 (Lai et al., 1984; Spaan et al., 1983) and also by sequencing of cDNA clones (Armstrong et al., 1984; Spaan et al., 1983). Because of the presence of 15 homologous nucleotides between the 3′-end of the leader RNA and the start site of mRNA No. 7 on the MHV genome (Fig. 1), it has been suggested that the splice junction lies within a stretch of 15 nucleotides (AAUCUAAUCUAAACU) (nucleotides 58–72 from the 5′-end) (Lai et al., 1984; Spaan et al., 1983). However, the exact length of the leader-coding sequences at the 5′-end of the genomic RNA is not clear. To determine the size of the leader-coding sequences on the genome, we purified the 72-nucleotide leader-specific cDNA made from mRNAs 6 and 7, using the previously described synthetic 15-mer as a primer for reverse transcription (Lai et al., 1984). This leader cDNA was hybridized to purified 60S genomic RNA and digested with S_1 nuclease. No alteration in the size of the 72-nucleotide cDNA was observed (Fig 2). The minor DNAs seen in Lane A were probably due to random degradation, or the presence of contaminating truncated leader cDNAs, since longer exposure revealed similar patterns in the undigested leader cDNA control (Lane B). However, minor heterogeneity of genomic RNA cannot be ruled out. The leader cDNA was completely digested by S_1 when it had not been hybridized to the virion RNA (Fig. 2C). These data indicate that a minimum of 72 bases encode the leader RNA at the 5′-end of the genome. However, the exact length of the leader RNA is not known.

Characterization of MHV leader-containing RNAs

We had previously proposed a model of coronavirus mRNA transcription which involved the synthesis of a free leader RNA (Baric et al., 1983). Since the experiments described above indicate that a minimum of 72 nucleotides at the 5′-end of the genome encode the leader RNA, the hypothetical free leader RNA synthesized

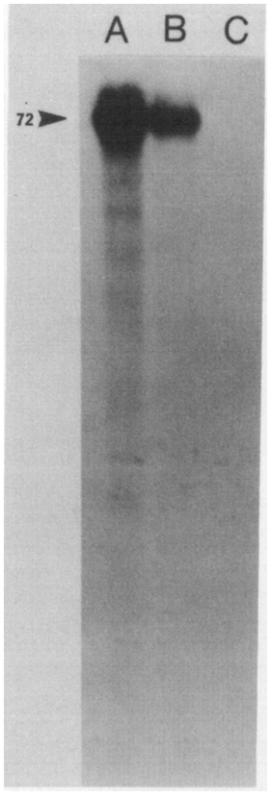

Fig. 2. S_1 mapping of leader-coding sequences on genomic RNA. Internally ^{32}P-labeled leader-specific cDNA (72 nucleotides long) was synthesized from mRNAs 6 and 7 and purified prior to hybridization with genomic RNA under the conditions described in the text. After hybridization, the hybrid was digested by S_1 nuclease and analyzed on 12% polyacrylamide gels containing 6 M urea. Lane A: S_1-digested leader/genome hybrid. Lane B: Undigested control. Lane C: Non-hybridized leader cDNA with S_1 nuclease.

during infection should also be of similar length. Initial attempts to identify this hypothetical RNA species, labeled in vivo, were unsuccessful, probably due to high backgrounds of tRNA and other small RNAs in infected cells. Furthermore, the free leader RNA was probably present in very small quantities since it would be expected to be constantly sequestered to prime transcription. To increase the sensitivity of detection, cDNA specific for the leader sequences was used as a probe to detect the presence of free leader RNA in infected cells. Intracellular RNA was separated on 20% or 12% polyacrylamide gels containing 6 M urea and electroblotted to Zeta-probe paper for Northern blot analysis. Under these conditions, nucleic acids smaller than 250 nucleotides in length enter the gel and are transferred efficiently. As shown in Fig. 3A, B, a small RNA species of approximately 75 nucleotides in length was detected with the leader-specific probe in infected, but not uninfected, cells. The size of this leader RNA agrees with that predicted from the S_1 mapping studies, although

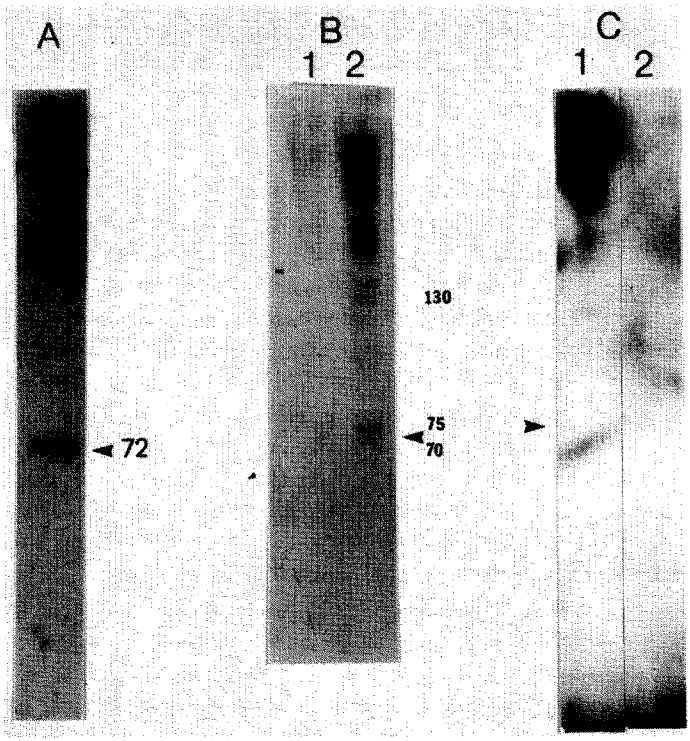

Fig. 3. Identification of leader-containing RNAs in MHV-infected cells. Intracellular RNA was harvested from A59-infected cells at 6 h post-infection (at 37°C) and separated on 12% or 20% polyacrylamide gels containing 6 M urea. The RNA was electroblotted to Zeta probe paper and probed with in vitro synthesized 5′-end-labeled leader cDNA (A, B) or a nick-translated cDNA clone (clone 143) (C). (A) RNA from MHV-infected cells (4 h p.i.) separated on 20% polyacrylamide gels. (B) Different preparations of RNA from uninfected (Lane 1) and infected cells (Lane 2) separated on 12% polyacrylmide gels. (C) RNAs from infected (Lane 1) and uninfected cells (Lane 2) probed with cDNA clone 143, which represents the first 800 nucleotides from the 3′-end of the genome. The arrows denote the positions of the in vitro synthesized 72-nucleotide leader-specific cDNA.

it is slightly larger. This result is consistent with the model that the leader RNA is synthesized as a separate entity which is discontinuous from the synthesis of the body sequences of mRNAs.

In addition to the 75-nucleotide leader RNA species, MHV-infected cells frequently contain multiple leader-related RNA species of different size (Fig. 3A, B). Usually a group of 2–4 species of leader RNAs in the range of 70–82 nucleotides were detected (see also Figs. 4 and 6). The larger leader-containing RNAs, ranging from approximately 130 to 250 nucleotides in length, were more heterogeneous and varied from preparation to preparation. Since similar RNA species were not detected by a cDNA probe (clone 143) which represents the first 800 nucleotides from the 3′-end of the genome (unpublished observation) (Fig. 3C), these data suggest that they were not derived from degradation of viral RNAs. The presence of these RNAs,

which are larger than the 70–82-nucleotide leader RNA, lends further support to a model of mRNA synthesis in which the leader RNA is utilized as a primer, rather than added post-transcriptionally (Baric et al., 1983).

Subcellular location of the MHV leader-containing RNA

The data described above suggests that leader RNA is synthesized as a separate entity which is discontinuous from the synthesis of the mRNAs. It is not clear, however, whether the leader RNA is released from the (−)-strand RNA template into a cytoplasmic pool, or remains tightly complexed with the RNA polymerase and/or (−)-strand template. To distinguish between these two possibilities, two approaches were undertaken. The first was to determine the subcellular localization of the leader-containing RNAs. It has previously been shown that RNA synthesis takes place in membrane-associated complexes during MHV infection (Brayton et al., 1982, 1984). Therefore, distribution of the leader RNA in different subcellular compartments should elucidate the mechanism of transcription involving the leader RNA species. MHV-infected cells were disrupted and separated into nuclear, cytosol (S-100) and membrane (P-100) fractions (Stohlman et al., 1983). RNA from each fraction was separated on polyacrylamide gels and probed for leader-specific RNA sequences (Fig. 4). All of the leader-containing RNAs were present in both the membrane and cytosol fractions while none were detected in the nuclear fraction. It is noteworthy that the leader RNAs in the range of 70–82 nucleotides were detected in roughly equimolar amounts in the cytosol and membrane fractions while the larger leader-related RNAs (greater than 130 nucleotides) were detected predominantly in the cytosol. This result is consistent with the interpretation that part of the 70–82-nucleotide leader RNA species is coupled to the membrane-associated transcriptional complex and involved in the synthesis of each mRNA. Moreover, although we can not completely rule out the possibility that our fractionation procedures might have disrupted the replication complexes, the detection of a significant portion of these RNA species in cytosol suggests that the leader RNA might also be present in a cytoplasmic pool.

We have studied the effects of temperature at which the virus is grown, with the purpose of further uncoupling the synthesis of leader-containing RNAs from that of mRNAs. Infected cells maintained at 40°C (Lane 4 in Fig. 4) appear to contain less leader RNA species than those grown at 37°C (Lane 5). This difference roughly corresponded to the general levels of RNA synthesis at these two temperatures (data not shown). Therefore, the synthesis of leader RNA and that of mRNAs could not be uncoupled at these two temperatures.

To determine whether the leader-containing RNAs are associated with the RNA template, intracellular RNA from infected cells was extracted and separated by sucrose gradient sedimentation to determine the size of RNA fractions in which leader RNA was detected. The majority of the leader-containing RNAs were found in 4S RNA fractions (Fig. 5). Since the larger-size RNA fractions do not contain distinct leader-related RNA species (Fig. 5), these results suggest that at least some leader RNA species are dissociated from the RNA replicative intermediate, which is 12–32S in size (Baric et al., 1983). However, this conclusion has to be qualified since

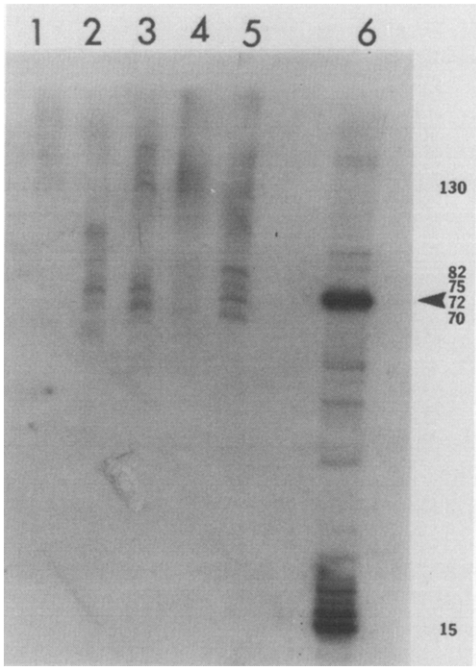

Fig. 4. Subcellular location of the MHV leader RNA. MHV-infected DBT cells were swollen in 0.5 × RSB buffer for 30 min at 6 h post-infection. The cells were disrupted and separated into nuclear, cytoplasmic (S-100) or membrane (P-100) fractions as described. Intracellular RNA was isolated from each fraction and separated on 12% polyacrylamide gels containing 6 M urea. Following transfer to Zeta-probe paper, the blots were hybridized with ^{32}P-labeled leader-specific cDNA. Lane 1: Purified nuclear fraction. Lane 2: Purified membrane fraction, P-100. Lane 3: Purified cytosol (S-100). Lane 4: A59-infected cells at 40°C. Lane 5: A59-infected cells at 37°C. Lane 6: In vitro synthesized leader cDNA as markers. The arrow denotes the 72-nucleotide leader cDNA.

we can not rule out the possibility that the leader RNA might have been dissociated from the replicative intermediate during the extraction procedure. These data are consistent with the subcellular fractionation studies which suggest that the leader-related RNAs are synthesized as a free entity and may function in viral transcription on membranes.

Identification of temperature-sensitive mutants which accumulate leader RNA at non-permissive temperature

The detection of free leader-containing RNAs in MHV-infected cells provides support for a mechanism of coronavirus transcription which utilizes a small leader RNA as a primer for mRNA synthesis. However, definitive proof of a distinct leader-specific RNA is complicated by the presence of multiple small leader-containing RNAs in infected cell extracts. To provide additional evidence for leader-primed transcription, we reasoned that the synthesis of leader RNA and mRNAs should be discontinuous and, therefore, might require different viral functions. Thus, it might

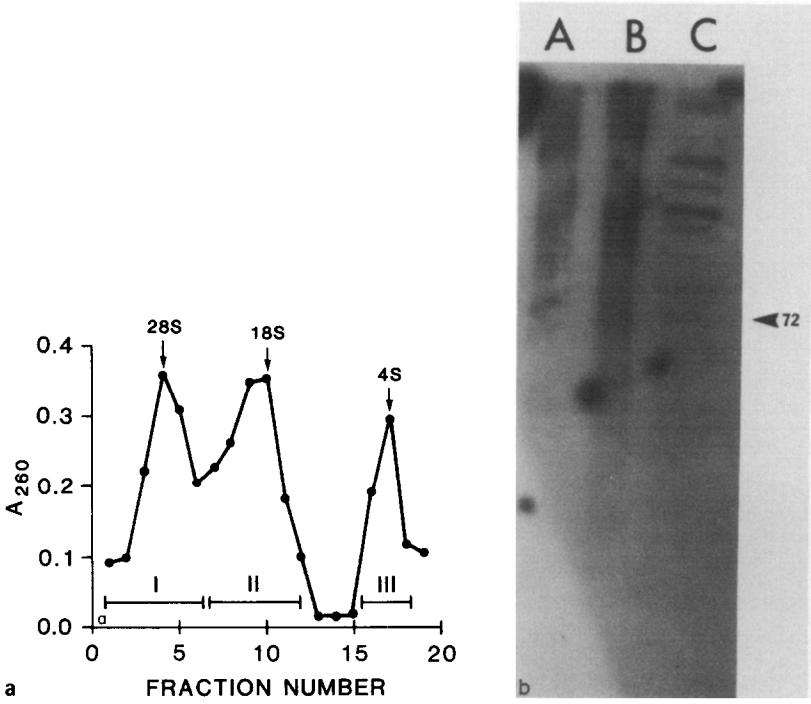

Fig. 5. Association of free leader RNAs with small RNA fractions in infected cells. Intracellular RNA was extracted from infected cells at 6 h post-infection and sedimented at 25000 rpm on 10–25% sucrose gradients for 16 h in an SW41 rotor. The gradients were pooled into 28S, 18S and 4S fractions (a) and electrophoresed on polyacrylamide gels as described in the text. Following transfer, the blotting paper was probed with the ^{32}P-labeled leader-specific cDNA (b). Lanes A–C represent fractions I to III, respectively. The arrow denotes the in vitro synthesized 72-nucleotide leader cDNA.

be possible to isolate a temperature-sensitive (ts) mutant which synthesizes only leader RNA but not mRNAs. We therefore examined several ts mutants of MHV for their ability to synthesize leader RNA at nonpermissive temperature. The majority of these ts mutants are RNA-(−), and fail to synthesize mRNAs later in the infection. These mutants also grow significantly more slowly than the wild-type virus at nonpermissive temperature (data not shown). Cells were infected with different ts mutants and incubated at permissive temperature for the first 6 h to permit synthesis of (−)-stranded RNA. The cultures were then shifted to nonpermissive temperature prior to the onset (+)-strand synthesis and analyzed for the presence of leader RNA 4 h later. Most of these ts mutants failed to synthesize any detectable RNA when probed with the leader-specific cDNA (Fig. 6). This is consistent with the observation that these mutants fail to synthesize mRNA at nonpermissive temperature (unpublished observation). However, one mutant, LA10, synthesizes a series of small leader-related RNA species at the nonpermissive temperature. The largest RNA had a similar size to the 72-nucleotide leader cDNA marker, although several smaller leader-containing RNAs were also detected. Since we did not detect larger leader-re-

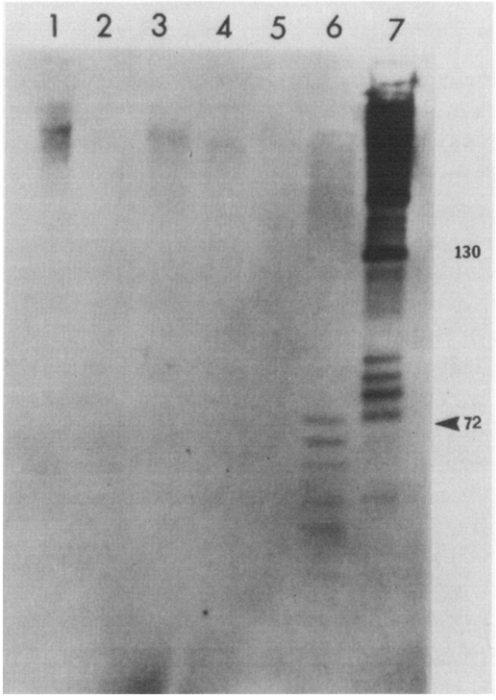

Fig. 6. Analysis of leader RNA synthesis with A59 ts mutants at the restrictive temperature. Cultures of L2 cells were infected with a variety of ts mutants of A59. Following 1 h for virus adsorption, the medium was replaced and the cultures incubated at 32°C for 6 h prior to shift-up to 39°C (nonpermissive temperature). Intracellular RNA was extracted at 10 h post-infection, and electrophoresed on 20% polyacrylamide gels. After electrotransfer to Zeta probe paper, the blots were probed with leader-specific cDNA. Lanes 1–7: LA1, LA2, LA5, LA6, LA8, LA10, XA9. The arrow denotes the 72-nucleotide leader cDNA.

lated RNAs, these data indicate that the replication of this mutant is blocked at a step after the synthesis of this 72-nucleotide leader RNA. They also suggest that this RNA species is not a premature termination product of mRNA synthesis, and that its synthesis is discontinuous from the synthesis of mRNA. The phenotype of LA10 is RNA-(−), as determined by rates of RNA synthesis at permissive and nonpermissive temperatures later in infection. Temperature-shift experiments also demonstrated that this mutant is defective in early steps of viral replication (data not shown). We have also examined an RNA-(+) mutant, XA9, which synthesizes the larger leader-containing RNA species, identical to those observed for wild-type virus, at the nonpermissive temperature (Fig. 6, lane 7). These results are consistent with the RNA-(+) phenotype of this mutant.

These results strongly suggest that the synthesis of the leader RNA and mRNAs requires separate viral functions. The presence of a mutant which accumulates leader-specific RNA at nonpermissive temperature lends further support to the model that the leader RNA is synthesized independently and then utilized as a primer for the synthesis of mRNAs.

Discussion

Previous studies on MHV mRNA structure indicate that each viral mRNA is constructed by the joining of two noncontiguous RNA segments encoded on the genome (Baric et al., 1983; Lai et al., 1983, 1984; Spaan et al., 1983). Since MHV mRNA synthesis occurs in the cytoplasm and does not involve synthesis and subsequent cleavage of precursor RNAs, viral transcription must occur by a novel mechanism that differs from conventional splicing modes (Baric et al., 1983; Lai et al., 1983; Jacobs et al., 1981; Spaan et al., 1983). Analysis of MHV replicative intermediate and replicative form RNAs supports this contention and suggests that a free leader RNA is transcribed independently, and then used to prime mRNA synthesis (Baric et al., 1983). In this publication, we have demonstrated the presence of multiple leader-containing RNAs in MHV-infected cells. Furthermore, we have identified a ts mutant which accumulates small leader RNAs at nonpermissive temperature. These findings provide strong evidence that MHV leader RNA synthesis is separate from the transcription of the mRNAs and provide additional support for the leader-primed mechanism of coronavirus transcription which we proposed previously (Baric et al., 1983).

Sequence analysis of mRNAs 7 and 6 suggests that the junction sites between the leader RNA and the body sequences of each mRNA lie between nucleotides 58 and 72 from the 5′-end of the genome (Armstrong et al., 1984; Spaan et al., 1983). From S_1 mapping studies, the length of the leader-coding sequences at the 5′-end of the genome is at least 72 nucleotides, but the exact length is not known. The major free leader RNA species identified in the MHV-infected cells is approximately 75 nucleotides in length. However, these data were complicated by the presence of several additional discrete leader-specific RNAs which range between 70 and 82 nucleotides in length. Several explanations could account for the multiple leader-related RNAs. Analysis of the primary sequence of the leader RNA suggests that several double-stranded RNA loops, similar to tRNA structure, may be present in the leader RNA (unpublished observation). These loops could represent strong stops for RNA synthesis, causing the accumulation of discrete premature termination products in vivo. Alternatively, the different leader RNAs might be primers for transcription of the different viral mRNAs. It has previously been suggested that differences in the degree of complementarity between the 3′-end of leader RNA and different junction sequences on the (−)-strand could regulate the rate of synthesis of each mRNA species (Fig. 1) (Baric et al., 1983; Spaan et al., 1983). The requirement for different leader RNAs might also provide an additional mechanism for the regulation of transcription of different mRNA species. The leader-primed transcription model is further supported by our recent finding that the leader sequences could be exchanged at very high frequency between two different MHV strains during mixed infection (Makino et al., unpublished observation). These data provide strong evidence for a mechanism of MHV transcription involving a free leader RNA. However, definitive proof that these leader RNAs act as primers awaits an in vitro transcription assay.

The larger leader-related RNA species which are longer than 130 nucleotides

might represent intermediate RNA products caused by premature termination of transcription. The fact that these RNA species contain the leader sequences and are significantly larger than the 75-nucleotide leader RNA provides further evidence that the leader RNA is utilized as a primer for transcription. It is conceivable that these intermediate-sized leader-related RNAs also participate in the process of transcription, i.e. they can bind back to the RNA template and act as primers for transcription. This possibility has recently been supported by the isolation of recombinant RNA molecules during mixed infection of different MHV strains (unpublished observations).

The uncoupling of leader RNA synthesis from mRNA synthesis with ts-LA10 provides strong evidence that transcription of MHV mRNAs requires more than one viral gene function. It is possible that two different polymerases are responsible for the synthesis of the leader RNAs and mRNAs, respectively. Alternatively, a specific viral protein might be required to either bind the leader RNA to the RNA template or modify RNA polymerase to allow the transcription of body sequences of mRNAs. Subunit regulation of a core polymerase has been proposed for the synthesis of alphavirus subgenomic mRNAs (Sawicki et al., 1978).

The 'leader-primed' mechanism for MHV RNA synthesis is a feature unique to coronaviruses. Several other viruses, such as VSV, a rhabdovirus, and La Crosse virus, a bunyavirus, also synthesize free leader RNAs (Colonno and Banerjee, 1976; Patterson et al., 1983). However, these leader RNAs are not joined covalently to the mRNAs. While the function of the bunyavirus leader RNAs has not been determined, the 47-nucleotide VSV leader is transported to the nucleus and serves as an inhibitor of host transcription (Grinell and Wagner, 1984; Kurilla et al., 1982; McGowan et al., 1982). Similar functions have not been detected with coronavirus leader RNA. On the other hand, the transcription of coronavirus partially resembles that of influenza virus. Although influenza virus does not synthesize a free leader RNA, it requires a host-derived 5′-capped RNA as a primer for transcription (Bouloy et al., 1978; Plotch et al., 1981). The leader-primed mechanism for RNA transcription employed by coronaviruses might have even more general occurrence. It has been shown that the mRNAs of the variable surface glycoprotein (VSG) in African trypanosomes contain an identical 37-nucleotide stretch at their 5′-ends (Boothroyd and Cross, 1982; Longacre et al., 1983). More recent studies have shown that a free leader RNA is synthesized which might serve as the primer for the VSG mRNA (Campbell et al., 1984; Milhausen et al., 1984). Thus, this leader-primed RNA transcription might represent a common alternative mechanism for generating spliced mRNAs in eukaryotic organisms.

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