An *in vitro* system for the leader-primed transcription of coronavirus mRNAs

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We have developed an *in vitro* transcription system which can utilize exogenous leader RNA for mouse hepatitis virus (MHV) 'leader-primed' mRNA transcription. Cytoplasmic extracts containing viral proteins and template RNA were prepared by lysolecithin permeabilization of MHV-infected cells. Synthetic leader RNA which differed in sequence from the endogenous leader RNA was added to the extracts and demonstrated to be incorporated into MHV mRNAs. Irrespective of the size of leader RNAs added, the exogenous leader RNA was joined to the endogenous mRNA at the same site, which corresponds to a UCUAA pentanucleotide repeat region. Only leader RNAs containing the pentanucleotide sequences could be utilized for transcription. Mismatches between the intergenic site and the exogenous leader sequence within the pentanucleotide repeat region were corrected in the *in vitro* system. This *in vitro* system thus established a novel mechanism of leader-primed transcription using exogenous RNA *in trans*, and suggests the involvement of a specific ribonuclease activity during coronavirus mRNA synthesis.

Key words: coronavirus mRNAs/free leader RNA/leader-primed transcription/*in vitro* transcription.

Introduction

Many cellular and viral RNAs are generated by discontinuous transcription from a template and contain sequences which are derived from discontinuous areas of the genome. For example, the VSG genes of *Trypanosoma brucei* (Murphy *et al.*, 1986; Sutton and Boothroyd, 1986), the actin gene of *Caenorhabditis elegans* (Krause and Hirsh, 1987) and rRNA genes of tobacco chloroplast (Koller *et al.*, 1987) utilize a *trans*-splicing mechanism to bring together RNA sequences that are generated as separate transcription units. On the other hand, influenza virus (Plotch *et al.*, 1981) and bunyavirus (Patterson *et al.*, 1984) utilize a 'cap-snatching' mechanism to use the 5'-end of a cellular RNA to prime transcription of viral mRNAs. Another extreme example of the discontinuous transcription is RNA editing. For example, some mitochondrial mRNAs of trypanosomatids use extragenic 'guide' RNAs to modify the nascent RNA transcript, so that the mature mRNAs contain sequences which are derived from other parts of the genome (Blum *et al.*, 1990).

An additional unique mechanism of discontinuous transcription is the hypothetical 'leader-primed' transcription

of coronaviruses (Lai *et al.*, 1984). This mechanism is proposed to involve the synthesis of a leader RNA which is used as a primer for the synthesis of subgenomic mRNAs. The murine coronavirus, mouse hepatitis virus (MHV), has been studied most extensively as a model system of leader-primed transcription. MHV contains a single-stranded infectious RNA genome of 32 kb (Pachuk *et al.*, 1989). In infected cells the virion RNA is first transcribed into negative-stranded RNA, which then serves as the template for a genomic size mRNA and six or seven subgenomic mRNAs (Figure 1A). These mRNAs have a 3'-coterminal, nested-set structure (Lai *et al.*, 1981; Leibowitz *et al.*, 1981) and each mRNA contains an identical 5'-end leader sequence of 72–77 nucleotides (Lai *et al.*, 1983, 1984; Spaan *et al.*, 1983). Only the 5'-end open reading frame (ORF) of each mRNA is translated, although these mRNAs contain multiple ORFs. In the intergenic regions between ORFs exists a stretch of sequence varying from 7 to 18 nucleotides which are homologous to the 3'-end of the leader RNA. This region presumably is involved in the regulation of transcription of mRNAs. There is a significant correlation between the extent of sequence homology between the intergenic region and the 3'-end of the leader RNA and the relative abundance of each mRNA (Shieh *et al.*, 1987), although this correlation is not observed in other coronaviruses, e.g. avian infectious bronchitis virus (Konings *et al.*, 1988). The structure of the negative-strand RNA template for these mRNAs is less certain. Both genomic and subgenomic sized negative-stranded RNAs have been detected and implicated in mRNA transcription (Lai *et al.*, 1982; Baric *et al.*, 1983; Sethna *et al.*, 1989; Sawicki and Sawicki, 1990). The mechanism of genesis of the subgenomic negative-strand RNA template has not been resolved.

Irrespective of the size of RNA templates, a considerable body of evidence suggests that the transcription of mRNAs involves a free leader RNA species, which is probably used as a primer for transcription (leader-primed transcription) (Baric *et al.*, 1983; Lai *et al.*, 1984). This mechanism would represent a unique mechanism of discontinuous transcription. The supporting evidence for this model includes: (i) Detection of 'free' leader RNA species of 50–90 nucleotides in the cytoplasm of MHV infected cells (Baric *et al.*, 1985, 1987); (ii) Isolation of a temperature sensitive mutant of MHV which makes only leader RNA but not mRNAs at the non-permissive temperature, suggesting that the syntheses of leader RNAs and mRNAs are discontinuous and require different viral proteins (Baric *et al.*, 1985); (iii) During mixed infection with two different MHV strains, the leader RNAs can be freely exchanged between the co-infecting viruses, again suggesting that the leader RNA exists as a separate transcription unit, independent of the viral mRNAs (Makino *et al.*, 1986); (iv) Each mRNA species of MHV is heterogeneous, varying in the number of a UCUAA repeat sequence present at the 3'-end of the leader RNA (Makino *et al.*, 1988b), which is consistent with this region being the
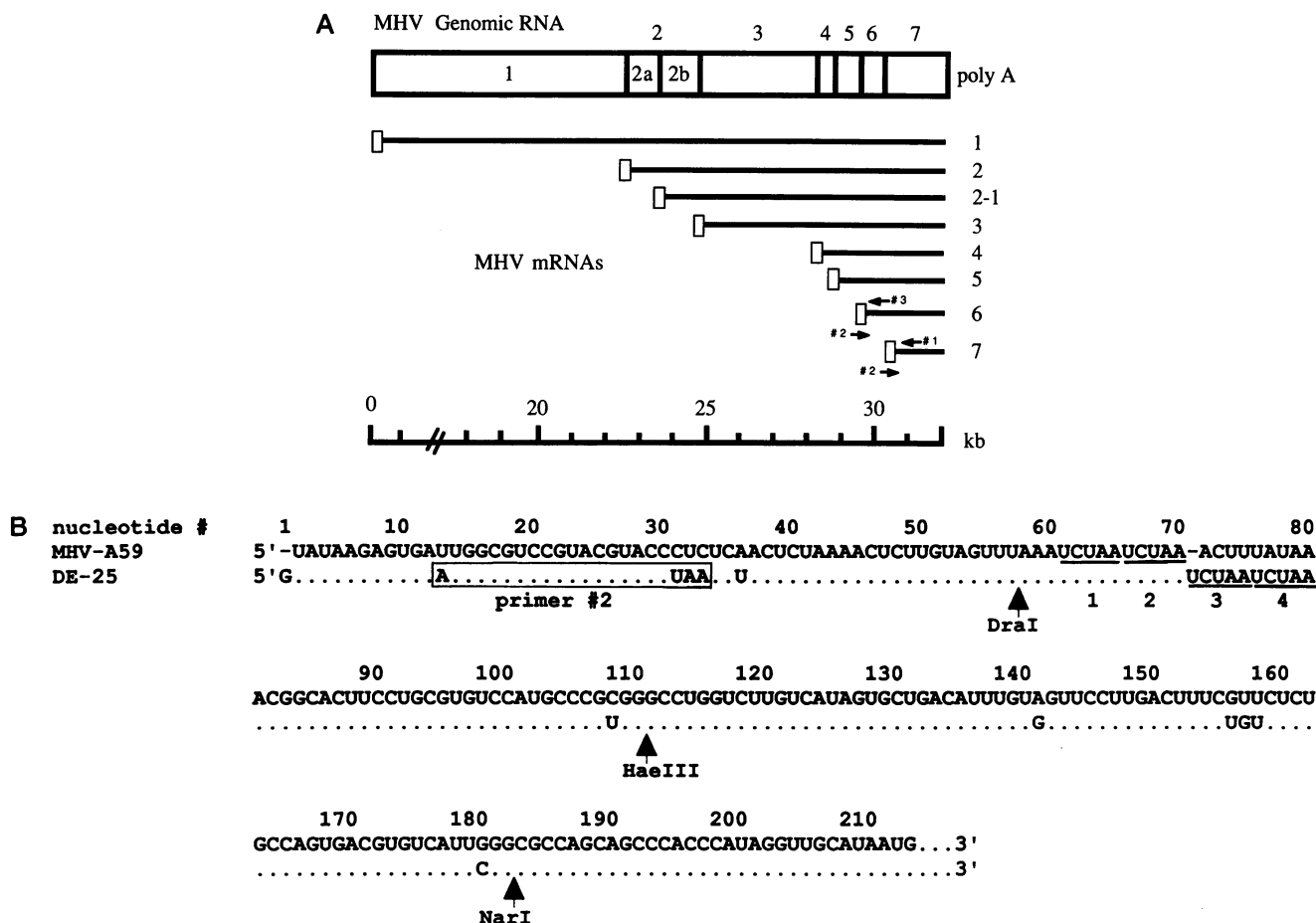
A MHV Genomic RNA

MHV mRNAs

B nucleotide #

Fig. 1. Structure of 5'-end sequence of coronavirus RNA. (A) Structure of the coronavirus genomic RNA and nested-set mRNAs. The 5' leader sequence of ~72 nucleotides is indicated by the open box. Location of the synthetic oligodeoxyribonucleotide probes are indicated by arrows. (B) Sequence comparison of the 5'-end region of MHV-A59 and DE-25 RNAs. The DE-25 sequence represents RNA sequence transcribed by T7 RNA polymerase. Sequence differences between DE-25 RNA and MHV-A59 are indicated whereas homologous sequence is specified by dots. The sequence used for the DE-25 leader specific probe (primer #2) is boxed. The pentanucleotide repeat region, UCUAA is underlined. Restriction enzyme sites used to linearize plasmid DNA before transcription are indicated by arrows.

leader RNA binding site; (v) The amount of an mRNA, 2-1, in different MHV strains is dependent on the 3'-end sequence of the leader RNA, suggesting that coronavirus mRNA transcription is the result of interactions between the 3'-end of the leader sequence and the complementary penta-nucleotide initiation sequence in the template RNA (Shieh *et al.*, 1989).

However, to date, there has been no direct evidence to corroborate the proposed leader-primed transcription of coronavirus. In this study, we present the first direct evidence of the utilization of free leader RNA in mRNA transcription. We have developed an *in vitro* transcription system which supports the utilization of exogenously added leader RNA. Furthermore, the exogenously added leader RNA appears to be trimmed at specific nucleotide mismatches, suggesting the involvement of a specific ribonuclease in coronavirus leader-primed transcription.

Results

Development of an in vitro transcription assay

To develop an *in vitro* system to demonstrate leader-primed transcription, we adapted the protocol of lysolecithin treatment of virus infected cells (Peluso and Moyer, 1983). This procedure involves the permeabilization of MHV A59

infected cell monolayers by treatment with lysolecithin. A reaction buffer containing nucleoside triphosphates was added and the cells were then scraped from the dish. After a brief centrifugation to remove nuclei and cell debris, the resultant supernatant was a cell-free extract containing the viral template RNAs and proteins required for transcription and replication (Carlsen *et al.*, 1985; Baker and Moyer, 1988). Since the amount of the MHV specific RNA polymerase is very low in the infected cells (data not shown), we added rabbit reticulocyte lysates to the *in vitro* system to increase the abundance of viral proteins necessary for transcription. Additionally, canine pancreatic membranes were added since coronavirus RNA synthesis has been shown to be associated with membranes (Brayton *et al.*, 1982, 1984). Indeed, *de novo* viral protein synthesis could be detected in this extract (data not shown). The addition of rabbit reticulocyte lysates and canine membranes increased viral RNA synthesis as monitored by [^{32}P]UTP incorporation into virus specific mRNAS (data not shown).

As a first step toward demonstration of leader-primed transcription, we examined whether an exogenously added free leader RNA could be utilized *in vitro*. As a source of free leader RNA, we used the leader RNA derived from a defective interfering RNA of coronavirus, DIssE (Makino *et al.*, 1988a). DIssE RNA was cloned and inserted
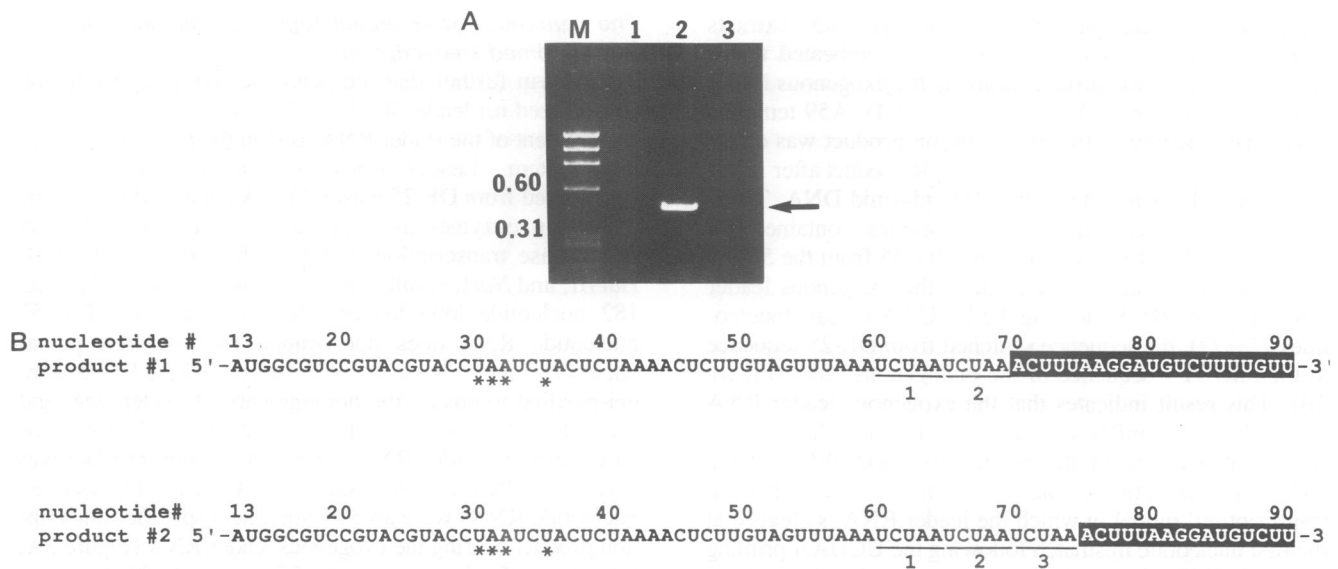
Fig. 2. Detection of the *in vitro* transcription products utilizing the exogenous leader RNA. (**A**) DE25 leader RNA of 111 nucleotides in length transcribed from the plasmid linearized with *Hae*III was added to cytoplasmic extracts from mock or MHV-A59 infected cells and *in vitro* transcription carried out for 2 h as described in Materials and methods. RNA was extracted and subjected to reverse transcription using a primer (# 1) specific for mRNA 7. The resultant cDNA was then amplified by PCR using primer #2 specific for the exogenous leader sequence. The PCR products were analyzed on a 1.5% agarose gel. Lane M: size marker φX174 DNA digested with *Hae*III, with relevant sizes indicated in kb on the left. PCR products from: mock infected extracts with exogenous leader (lane 1); MHV-A59 infected extract with exogenous leader (lane 2); MHV-A59 extract alone (lane 3). (**B**) Sequence of PCR amplified transcription products. The 466 nucleotide PCR product (lane 2 in panel B) was gel-purified, subcloned into pTZ-18U and sequenced as described in Materials and methods. The two types of leader/mRNA 7 junction region sequence are shown. The DE-25 specific nucleotides are identified by an asterisk, the UCUAA repeats are underlined and the mRNA 7 body sequence is highlighted.

downstream of a T7 RNA polymerase promoter, and this plasmid was designated DE-25 (Makino and Lai, 1989). The leader region (nucleotides 1−80) of DE-25 differs from that of the endogenous MHV-A59 at several positions (Figure 1B). Most notably, DE-25 contains four UCUAA pentanucleotide repeats at the 3' end of the leader sequence, whereas MHV-A59 contains only two UCUAA repeats (Makino and Lai, 1989; Lai *et al.*, 1984). These penta-nucleotide repeat regions are located at the junction between the leader RNA and mRNA body sequence. In addition, DE-25 contains five nucleotide differences within the first 35 nucleotides of the leader RNA. These nucleotide differences were exploited to distinguish endogenous mRNAs from those made with the exogenously added leader RNA. We developed a polymerase chain reaction (PCR) detection assay using synthetic primers which were specific for mRNA body sequences and the exogenous DE-25 leader RNA respectively. Specifically, we used a primer complementary to mRNA 7 (oligonucleotide # 1) to prime cDNA synthesis by reverse transcriptase. A second primer specific for DE-25 RNA and containing the three contiguous diverged nucleotides at its 3'-end (oligonucleotide #2) (Figure 1B) was used together with primer # 1 for PCR amplification. This system allowed the specific detection and amplification of RNAs which have exogenous leader RNAs joined to mRNA 7 body sequence.

Exogenous leader RNA primes transcription of mRNA 7

Plasmid DE-25 was linearized by *Hae*III and capped RNA synthesized using T7 RNA polymerase as described in Materials and methods. The resultant RNA was 111 nucleotides in length and contained four UCUAA penta-nucleotide repeats. This RNA was added to mock or MHV-
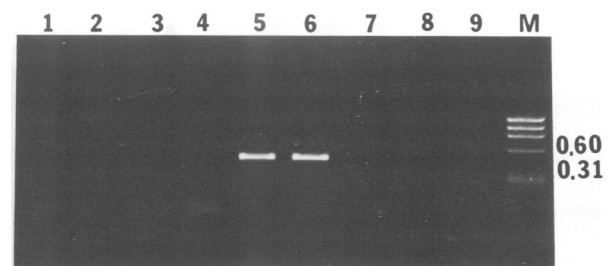

Fig. 3. Effect of leader length on *in vitro* leader-primed transcription of mRNA 7. DE-25 DNA was linearized by *Dra*I, *Hae*III or *Nar*I and RNA transcribed by T7 RNA polymerase resulting in RNAs of 57, 111 or 182 nucleotides, respectively. Gel-purified RNAs were added to cytoplasmic extracts, incubated *in vitro* and products analyzed by reverse transcription and PCR amplification as described in Figure 2. Lane M: size marker φX174 DNA cut with *Hae*III with the relevant sizes given in kb. PCR products from: mock-infected extracts with 57 nucleotide leader (lane 1), 111 nucleotide leader (lane 2), 182 nucleotide leader (lane 3); MHV-A59 extracts with 57 nucleotide leader (lane 4), 111 nucleotide leader (lane 5), 182 nucleotide leader (lane 6) or MHV-A59 extracts incubated alone, RNA extracted and subjected to reverse transcription and PCR amplification in the presence of the 57 nucleotide long leader (lane 7), 111 nucleotide leader (lane 8) or 182 nucleotide leader (lane 9).

A59 infected cytoplasmic extracts and incubated *in vitro* for 2 h at 30°C. The extracts were then treated with proteinase K and RNA extracted by phenol:chloroform. The RNA was subjected to reverse transcription using oligonucleotide # 1 and then amplified by PCR using oligonucleotide #2. A 466 nucleotide product was detected only in extracts of MHV-A59 infected cells to which exogenous DE-25 leader was added (Figure 2A, lane 2). This product was not present in either mock-infected extracts to which leader RNA had

been added (Figure 2A, lane 1) or in MHV-A59 extracts alone (Figure 2A, lane 3). These results indicated that a specific transcription product utilizing the exogenous leader RNA was made from the endogenous MHV-A59 template RNA. The identity of this transcription product was determined by sequencing the individual PCR product after it was cloned into the SmaI site of PTZ18U plasmid DNA. These sequences revealed that all of the clones contained the nucleotides UAAUCU at positions 30−36 from the 5'-end of the RNA, which are identical to the exogenous leader RNA (Figure 2B). Following the UCUAA repeats (nucleotides 72−77), the sequence switched from DE-25 sequence to the mRNA 7 sequence of the endogenous virus (Figure 2B). This result indicates that the exogenous leader RNA was utilized for mRNA 7 synthesis and that there was a specific processing of the exogenous leader RNA at the UCUAA sites. This is consistent with the 'leader-primed transcription' model in which the leader RNA is cleaved at the first nucleotide mismatch following the UCUAA priming site (Lai, 1988). Interestingly, sequencing also showed that there was heterogeneity in the number of UCUAA repeats in the mRNAs. The majority of the PCR product clones (36/39) have two pentanucleotide repeats at the junction site, but the remaining three clones contained three pentanucleotide repeats (Figure 2B). This result confirms the mRNA heterogeneity seen in the virus infected cells (Makino et al., 1988b), and is consistent with the interpretation that the UCUAA repeat region is the leader RNA binding site, and that the mode of binding is variable (see Discussion).

The pentanucleotide repeat region is essential for leader-primed transcription

To confirm further that the pentanucleotide repeat region was utilized for leader RNA binding, we examined the size requirement of the leader RNA used in the in vitro transcription system. Leader RNAs of various lengths were synthesized from DE-25 plasmid DNA linearized by specific restriction enzymes as indicated in Figure 1. T7 RNA polymerase transcription of DE-25 linearized with DraI, HaeIII, and NarI, resulted in the synthesis of a 57, 111 and 182 nucleotide long leader RNA, respectively. The 57 nucleotide RNA does not contain the UCUAA pentanucleotide region, while the other two do. These RNAs were gel-purified to ensure the homogeneity of leader size, and then added to mock or MHV-A59 infected cell extracts as an exogenous leader RNA. The transcription product was detected by PCR as described above. Only the 111 and 187 nucleotide RNAs were able to generate a specific transcription product bearing the exogenous leader RNA (Figure 3A, lanes 5 and 6). In contrast, the 57 nucleotide RNA, which does not have the UCUAA repeat, was unable to prime transcription (Figure 3A, lane 4). A minor band, which was < 100 nucleotides in size, was detected in lane 4. This band probably represents free oligonucleotide primer or nonspecific amplification products. No specific PCR products were detected in the mock infected cell extracts with added leader RNAs (Figure 3A, lanes 1−3) or in a mixture of the purified leader RNA and RNA from the MHV-A59 infected cells (Figure 3A, lanes 7−9). The latter served as a control
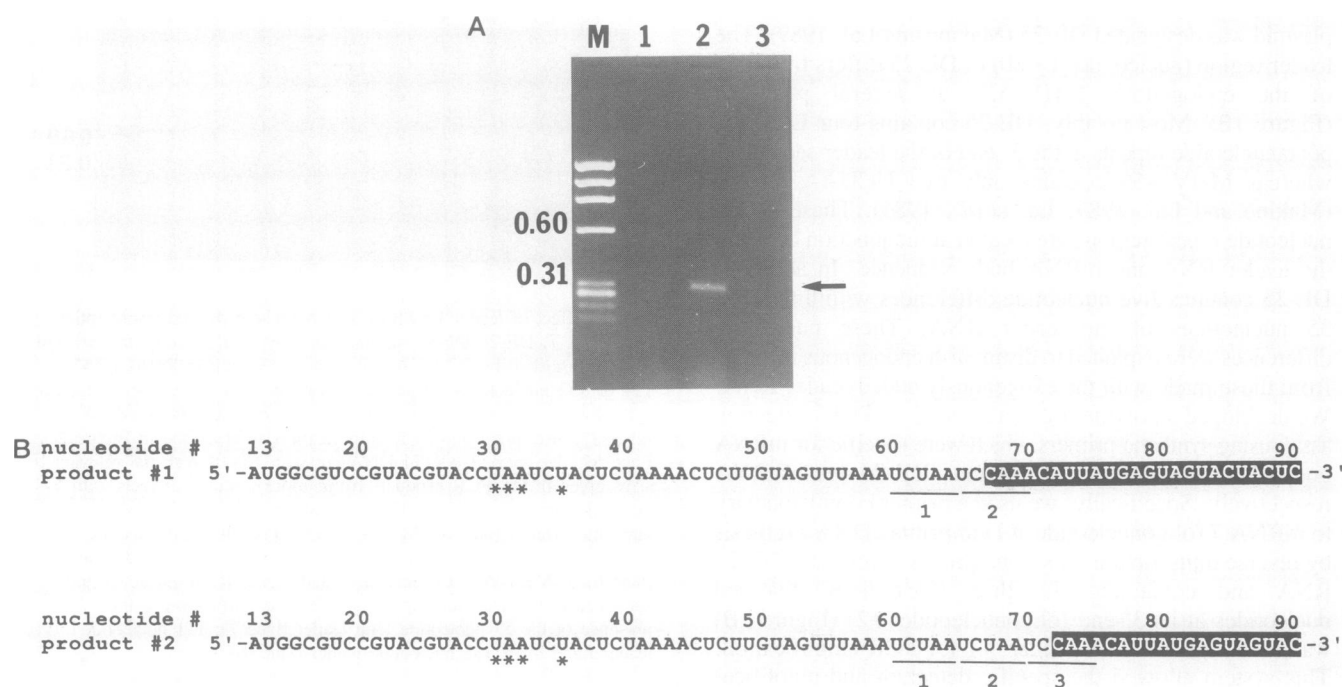

Fig. 4. In vitro transcription of mRNA 6 using exogenous leader RNA. (A) Exogenous leader RNA of 111 nucleotides in length was incubated with mock or MHV-A59 infected cell extracts as described in Figure 2. RNA was extracted and subjected to reverse transcription using oligonucleotide primer #3 specific for mRNA 6 and PCR amplification using primer #2 specific for the exogenous leader sequence. PCR products were analyzed on a 1.5% agarose gel. Lane M: size marker φX174 DNA digested with HaeIII. PCR products from: mock infected extracts incubated with exogenous leader (lane 1), MHV-A59 infected cell extracts incubated with exogenous leader (lane 2) and MHV-A59 infected cell extract alone (lane 3). (B) Sequence of the mRNA 6 exogenous leader specific PCR product. The 314 nucleotide long PCR product was gel-purified subcloned into pTZ18U and sequenced as described in Materials and methods. The two types of leader/mRNA 6 junction region sequence detected are shown. The DE-25 specific nucleotides are identified by an asterisk, the UCUAA and UCCAA repeats are underlined and the RNA 6 body sequence is highlighted.

for the fidelity of reverse transcription and PCR amplification. The sequence analysis of the cloned PCR products showed that the leader RNA of 180 and 111 nucleotides yielded exactly the same transcription products, in which the exogenous leader RNA switched to the endogenous mRNA sequence at precisely the UCUAA repeat, irrespective of the size of leader RNA used (Figure 2B). These results further established that the pentanucleotide region is required for transcription and that the leader RNA was trimmed at the leader RNA binding sites (UCUAA repeats), prior to transcription of the mRNA body sequence.

Leader-primed transcription recognized nucleotide mismatches

To determine precisely where the leader RNA was trimmed, we examined the transcription of mRNA 6. The intergenic junction of mRNA 6 contains an imperfect repeat of the pentanucleotide sequence, i.e. UCUAA UCCAA. Thus, the second repeat differs from the leader RNA sequence (UCUAA UCUAA) at the 5'-end of the genome (Makino et al., 1988b). We wanted to determine if the mRNA 6 transcription product contains the pentanucleotides of the exogenous leader RNA or the endogenous template RNA. Leader RNA of 111 nucleotides was used in in vitro transcription. An oligonucleotide primer specific for the body sequence of mRNA 6 (oligonucleotide #3) was then used for cDNA synthesis and primer #2 was used as the second primer for PCR amplification. A specific product of the expected 314 nucleotides was detected only in MHV-A59 infected cell extracts to which the exogenous leader RNA had been added (Figure 4A, lane 2). Sequencing of the PCR products showed that the mRNA 6 transcription product contains UCUAA UCCAA, the same as the template RNA (Figure 4B). This result implied that the leader RNA was trimmed at or before the site of the nucleotide mismatch between the leader RNA and intergenic site. There were also two populations of PCR clones, those with UCUAA UCCAA (i.e. two repeat) and UCUAA UCUAA UCCAA (i.e. three repeat) sequences. It is interesting to note that the UCCAA was always at the last pentanucleotide repeat, suggesting that the exogenous leader RNA downstream of the mismatch was not retained in the mature mRNA 6. The mRNA 6 clones with two repeats versus three repeats were almost equally abundant (11/23 are two repeat, 12/23 are three repeat). These results again suggest that the pentanucleotide repeat region is involved in the leader RNA binding during mRNA synthesis.

Discussion

The data presented in this study have established that an exogenous leader RNA can be incorporated in trans into the mature subgenomic mRNAs of coronavirus. This is the first direct biochemical demonstration of the role of a free leader RNA in coronavirus mRNA synthesis. Since coronavirus RNA replicates exclusively in the cytoplasm (Brayton et al., 1981; Wilhemsen et al., 1981) and it does not have a consensus splice donor and acceptor sequences, these data are inconsistent with the trans-splicing model, and strongly support the leader-primed transcription mechanism proposed previously (Lai, 1988). This system allowed us to establish several details of leader-primed transcription (Figure 5). In

this mechanism, the leader RNA is synthesized from the 5'-end of the template RNA, dissociates from the template and rebinds to the template RNA at the downstream transcriptional initiation sites. The 3'-end overhang sequence of the leader RNA is removed and transcription starts at the 3'-end of the trimmed leader RNA.

Several key components of this unique transcription mechanism are worth noting. (i) The UCUAA pentanucleotide repeat region at the 3'-end of the leader RNA is essential for transcription (Figure 3). This pentanucleotide region is complementary to the conserved intergenic region. This conclusion is supported by the findings that the leader RNA which did not include the UCUAA repeat could not be incorporated into mRNAs and that the number of UCUAA repeats is variable from clone to clone. The latter finding also suggests that the interaction between the leader RNA and the template RNA at this pentanucleotide repeat site is imprecise. Thus, the sequence complementarity between the leader RNA and the template RNA is likely the mechanism by which the leader RNA binds to the template RNA. (ii) The leader RNA appeared to be processed before being used for mRNA transcription. This conclusion is supported by the finding that the leader RNAs of 111 and 182 nucleotides yielded the same type of mRNA products. Thus the 3'-end sequences beyond the pentanucleotide repeat
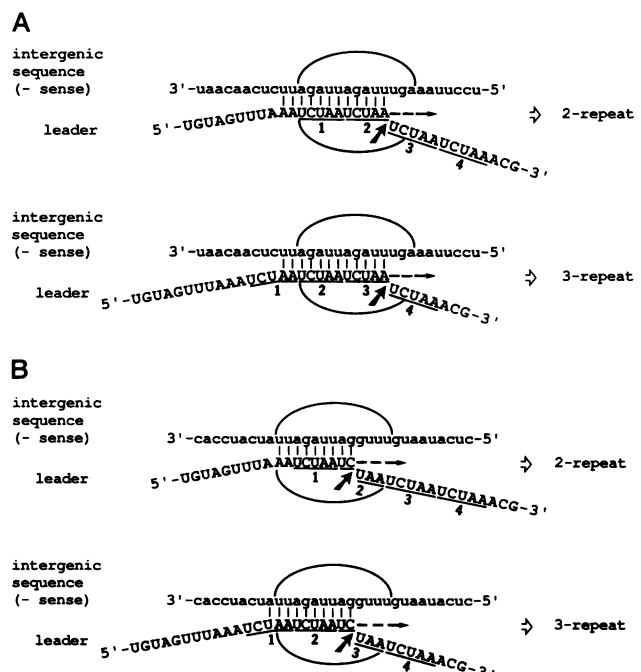

Fig. 5. Proposed mechanism of coronavirus leader-primed transcription. The leader RNA binds to the intergenic junction sites via the complementary pentanucleotide region of the template and acts as a primer for the transcription of each mRNA. (A) Alignment of leader RNA at the gene 6/7 intergenic site. The 3'-end of the leader RNA aligns with the homologous intergenic junction site. At the first base mismatch following the pentanucleotide repeat, the leader RNA is cleaved and mRNA transcription is initiated. Possible alignments for the generation of two repeat mRNA 7 and three repeat mRNA 7 are shown. The circle represents the binding domain of RNA polymerase. (B) Priming of MHV mRNA 6. The intergenic junction region between genes 5 and 6 contains an imperfect pentanucleotide repeat. The 3'-end of the leader RNA aligns and is cleaved at the site of the nucleotide mismatch within the intergenic region and mRNA transcription is initiated. The circle represents the binding domain of RNA polymerase.

4177

region appear to be inconsequential for mRNA synthesis. Since all of the mature mRNAs switched from the exogenous leader RNA sequence to the endogenous template RNA sequence at precisely the pentanucleotide repeat region, irrespective of the size of the leader RNA used, the 3′-end sequence of the leader RNA is likely removed by an exonuclease or endonuclease activity prior to RNA synthesis. Such a nuclease activity would probably remove only the single-stranded RNA sequence from the 3′-end of the leader RNA. This finding is consistent with the previous observation that the leader-containing RNAs detected in the MHV infected cells are larger than the 72 nucleotides present in the mature mRNAs (Baric et al., 1985). These larger leader RNAs can thus participate in mRNA synthesis after trimming of the 3′-end sequence. (iii) The mismatches between the 3′-end of the leader RNA and template RNA in mRNA 6 were corrected so that the sequence of the mature mRNA matches the template RNA rather than the exogenous leader RNA. Thus, the nuclease activity postulated above apparently would be able to recognize the mismatch within a stretch of perfectly matched, double-stranded region. This activity may be analogous to the RNA cleavage activity associated with some RNA editing systems (Blum et al., 1990). Alternatively, the nuclease activity may recognize a secondary or tertiary structure at the priming site, allowing for trimming of the primer and synthesis of the mature mRNA according to the negative strand template sequence.

This transcription mechanism is distinct from any of the discontinuous transcription mechanisms known so far. Influenza virus RNA synthesis is similar; however, the primer RNA used by influenza virus is not sequence specific. The influenza virus RNA polymerase has been shown to have an endonuclease activity to process the primer (Plotch et al., 1981). This activity does not recognize the specific nucleotide mismatch as proposed here for coronavirus RNA synthesis. The possible existence of such a nuclease activity associated with coronavirus RNA polymerase may lower the error frequency of RNA synthesis, thus allowing the 32 kb RNA genome, which is by far the largest genomic RNA, to be faithfully transcribed.

The template RNA for this leader-primed transcription is currently unknown. Our in vitro transcription system described here utilizes the endogenous template RNAs. It has been shown that both the genomic sized (Lai et al., 1982; Baric et al., 1983) and subgenomic negative strand RNAs (Sethna et al., 1989; Sawacki and Sawacki, 1990) are present in the coronavirus infected cell. The subgenomic negative-strand RNAs could be derived from the replication of the subgenomic mRNAs (Sethna et al., 1989). Both the genomic and subgenomic negative-stranded RNA can potentially be used as the templates for the leader-primed transcription. The precise sequence requirement for recognition of the negative-strand RNA awaits additional studies.

The proteins responsible for leader-primed transcription are also unknown. Presumably the virus-encoded RNA polymerases carry out this function. It has been shown that these proteins are encoded from a 22 kb gene at the 5′-end of the coronavirus genome (Boursnell et al., 1986; Gorbalenya et al., 1989; Pachuk et al., 1989). The size of this gene predicts a protein of >800 kd which is likely processed into several proteins by a proteolytic enzyme (Baker et al., 1989). Only the amino terminal p28 protein has so far been detected in virus infected cells (Denison and

Perlman, 1987). Probably because of the unstable nature of the polymerases, none of the other predicted protein products have been detected in virus infected cells. The use of rabbit reticulocyte lysate in our transcription system probably has overcome this problem and allowed us to detect leader-primed RNA transcription in vitro. The characterization of these proteins should reveal many interesting enzymatic properties.

Materials and methods

Virus and cell line

Mouse hepatitis virus strain A59 (MHV-A59) (Manaker et al., 1961) was grown in monolayer cultures of murine fibroblast 17C1-1 cells (kindly provided by Dr S.Sawicki, Medical College of Ohio). The 17C1-1 cells were maintained in Dulbecco's modified Eagle's medium with 5% tryptone phosphate broth and 3% newborn calf serum. The cells were infected with MHV-A59 at an m.o.i. of 10.

Preparation of exogenous leader RNA

Plasmid DE-25 has been described previously (Makino and Lai, 1989). This plasmid contains the complete MHV-DIssE sequence downstream of the T7 RNA polymerase promoter. The sequence of the 5′-end leader is shown in Figure 1B. DE-25 plasmid DNA was linearized by DraI, HaeIII or NarI digestion and capped RNA was transcribed with T7 RNA polymerase (US Biochemicals) as previously described (Soe et al., 1987; Tabor and Richardson, 1985) and added directly to MHV-A59 cytoplasmic extracts. When required, RNAs were purified on 6% polyacrylamide−7M urea gels using [^{32}P]CTP-labeled RNAs as markers. The region containing the RNA was excised from the gel and eluted overnight at room temperature in RNA extraction buffer (0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% SDS, 0.001 M EDTA), followed by phenol extraction and ethanol precipitation. The precipitated RNA (0.1 μg) was resuspended in 4 μl of water before addition to cell extracts.

In vitro transcription assay

Cytoplasmic extracts of 17C1-1 cells infected with MHV-A59 were prepared by the lysolecithin permeabilization procedure as described by Peluso and Moyer (1983). Briefly, monolayers of 17C1-1 cells (60 mm dish) were infected with MHV-A59 (m.o.i.=10) and cytoplasmic extracts prepared at 12 h post-infection. Cells were treated with 1 ml of 125 μg/ml lysolecithin (L-α-lysophosphatidylcholine, palmitoyl; Sigma Chemical Co., St Louis, MO) for 1 min at 4°C. The treated cells were scraped into 350 μl of a reaction mixture containing 0.1 M HEPES pH 8.0 with KOH, 0.25 M NH$_4$Cl, 7 mM KCl, 4.5 mM MgAc, 1 mM DTT, 1 mM spermidine, 1 mg/ml poly-L-glutamate, 1 mM each of ATP, CTP, UTP and GTP, creatine phosphokinase at 10 U/ml and 10 mM creatine phosphate. The cells were disrupted by pipetting 10 times with a Pasteur pipette and then centrifuged at 800 g for 3 min to remove nuclei and cell debris. The resultant cell-free supernatant fluid (200 μl per reaction) was mixed with 20 μl rabbit reticulocyte lysates (Promega), 4 μl canine pancreatic membranes (Promega) and 0.1−0.3 μg of DE-25 leader RNA (final reaction volume of 230 μl) and incubated at 30°C for 2 h. The addition of rabbit reticulocyte lysates and canine membranes stimulated the translation of labile proteins which may be required for transcription of coronavirus mRNAs. After incubation, the reaction mixtures were treated with proteinase K (500 μg/ml) for 30 min at 37°C. The RNA was extracted with phenol−chloroform and precipitated by addition of ethanol.

Polymerase chain reaction amplification of RNAs

One tenth (10 μl) of the RNA isolated from the in vitro transcription reaction was reverse transcribed into cDNA (AMV reverse transcriptase, Seikagaku America Inc.). Briefly, the RNA was denatured at 72°C for 10 min and then incubated at 37°C for 60 min in 50 μl of buffer containing 10 mM MgCl$_2$, 100 mM KCl, 50 mM Tris−HCl pH 8.3, 10 mM dithiothreitol, 0.1 mM each of deoxyribonucleoside triphosphates, 60 units of ribonuclease inhibitor RNasin (Promega), 1 μM synthetic oligonucleotide primer specific for MHV-A59 mRNA 7 (oligo #1: 5′-CCAAGATAGTAAAAATA-CCA-3′) or mRNA 6 (oligo #3: 5′-TTAGCGCATACACGCAATTG-3′). The cDNA was then utilized as the template for amplification by the method of Saiki et al., 1988; Briefly, 5 μl of reverse transcription mixture was mixed with 90 μl of buffer containing 50 mM KCl, 10 mM Tris−HCl pH 8.3, 1.25 mM MgCl$_2$, 0.01% gelatin, 100 μM each of deoxyribonucleoside triphosphates, 1 U of Taq polymerase (Cetus) and 0.5 μM of synthetic

oligonucleotide primer specific for the exogenous DE-25 leader RNA (oligo #2: 5'-TGGCGTCCGTACGTACCTAA-3'). Amplification was carried out for 30 cycles at 95°C (30 s), 55°C (30 s), and 72°C (1 min) per cycle. The PCR products were then analyzed on 1.5% agarose gels in 1× TBE buffer (0.089 M Tris−HCl, 0.089 M boric acid, 0.002 M EDTA) and stained with ethidium bromide.

Sequencing of PCR products

Subcloning and sequencing of the PCR products followed the methods by Sambrook et al. (1989). Briefly, products of the PCR amplification were separated and extracted from a 1.5% low-melting agarose gel, treated with polynucleotide kinase (Boehringer-Mannheim), blunted by 1 U of T4 DNA polymerase (Boehringer-Mannheim) at 37°C for 30 min in buffer containing 50 mM Tris−HCl pH 7.5, 7 mM MgCl, 1 mM DTT, 0.25 mM each of deoxyribonucleoside triphosphates followed by ligation into the SmaI site of pTZ-18U. The ligated DNA was transformed into Escherichia coli DH5α (BRL) and miniprep DNA prepared by the boiling method (Sambrook et al., 1989). Double-strand DNA sequencing was then performed using the Sequenase System (US Biochemicals).

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