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Coronavirus mRNA Synthesis: Identification of Novel Transcription Initiation Signals Which Are Differentially Regulated by Different Leader Sequences

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The mRNA synthesis of mouse hepatitis virus (MHV) has been proposed to be the result of interaction between the leader RNA and the intergenic sites. Previously, we have identified a transcription initiation site (for mRNA 2-1), which is more efficiently transcribed by viruses containing two copies of UCUAA sequence in the leader RNA than by those with three copies. In this study, we have identified several sites which are regulated in the opposite way, namely, they are efficiently transcribed by the leader RNA with three UCUAA copies but not by those with two copies. These sites were characterized by primer extension and amplification by polymerase chain reaction. One of these sites is in the gene 3 region of a recombinant virus between A59 and JHM strains of MHV. Another is in the gene 2 region of MHV-1 strain. Both of these sites have a sequence similar to but different from the consensus transcription initiation signal (UCUAAUCUAUC and UUUAUCUU, as opposed to UCUAAAC). These two novel intergenic sequences are not present in the genome of the JHM strain, consistent with the absence of these mRNAs in the JHM-infected cells. The discovery of this type of transcription initiation site provides additional evidence for the importance of the leader RNA in the transcription initiation of MHV mRNAs. © 1992 Academic Press, Inc.

Mouse hepatitis virus (MHV), a member of the Coronaviridae, is an enveloped virus containing a nonsegmented positive-sensed RNA genome of 31 kb (1, 2). MHV synthesizes seven to eight virus-specific mRNAs that have a 3'-coterminal nested-set structure (1, 3). The 5' unique portion of each mRNA is utilized for translation (4, 5). These mRNAs encode four structural proteins, including S (spike), translated from mRNA 3, N (nucleocapsid) from mRNA 7, M (membrane) from mRNA 6, and HE (hemagglutinin-esterase) from mRNA 2-1. Other mRNAs encode nonstructural proteins, which have not been well characterized [for review, see Ref. (1)]. mRNA 2-1 is unusual in that it is synthesized by only some, but not all, of the MHV strains (6); its presence or absence is apparently regulated by a stretch of leader sequence located at the 5'-end of the genomic RNA (7). This leader RNA sequence (approximately 72 nucleotides) is also found at the 5'-end of every MHV mRNA species (8).

At the 3'-end of the leader sequence, there are several copies of a repeat pentanucleotide sequence UCUAA, whose copy number varies from two to four, depending on the virus strain (9), and decreases upon serial passage of the virus *in vitro* (10). Significantly, a JHM virus which contains three copies of UCUAA in

the leader RNA makes only a small quantity of mRNA 2-1, while the same virus with two copies makes a large quantity of this transcript (7, 10). The amounts of some other mRNAs, e.g., mRNA 2 and 4, are also affected, but to a much smaller extent (7, 10).

The level of mRNA 2-1 expression can also be affected by the sequence at the transcription initiation site for this mRNA. For example, the A59 strain of MHV has a single base substitution within this transcription initiation site and does not synthesize mRNA 2-1, despite the presence of two UCUAA copies (7, 11). These results suggest that MHV mRNA synthesis is determined by the interaction between the leader RNA and the intergenic sites. Thus, the intergenic sequence for gene 2-1 may represent a unique class of sequence which is differentially regulated by leader RNAs with different UCUAA copy numbers. The basis for this differential regulation of transcription is not clear. To understand the mechanism of MHV mRNA transcription, we have attempted to determine whether the differential regulation of transcription initiation by leader RNA containing different UCUAA copy numbers is unique to mRNA 2-1. In this report, we have identified several additional transcription initiation sites which are regulated in different manners by leader RNA with different copy numbers of the UCUAA sequence. These studies provide further insight into the mechanism of MHV mRNA transcription.

We previously obtained a recombinant virus B1 between the A59 and JHM strains of MHV (12). This virus

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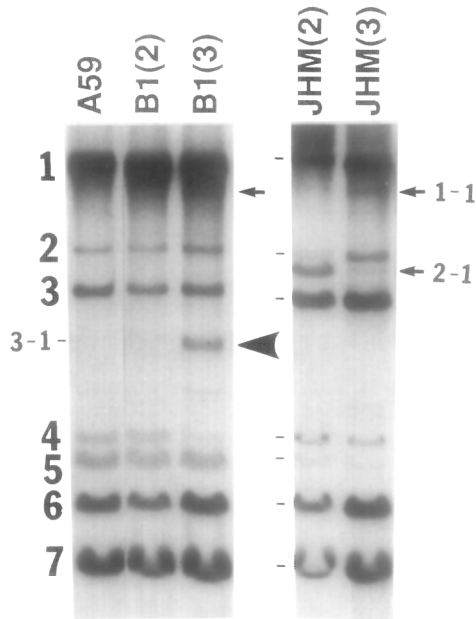


Fig. 1. Electrophoretic analysis of virus-specific intracellular RNAs of various MHV strains. Viruses were grown in mouse astrocytoma cell line DBT (17) and labeled with 0.2 mCi/ml of [32 P]-orthophosphate between 5 and 9 hr p.i. RNA was extracted from the infected cells, denatured with glyoxal and DMSO, and analyzed by electrophoresis on a 1% agarose gel as described (18).

has a genome structure in which the 5'-end of the genome, including the leader RNA and part of gene 1, was derived from JHM, while the remaining sequence was from A59 (12). This virus synthesized a novel mRNA 3-1 (previously named 3a), which was initiated within the gene 3 (12). By passaging B1 virus in tissue culture, a new virus isolate was obtained, which differed from the original B1 virus in that it had two copies of the UCUSA sequence in the leader, in contrast to the three copies in the original virus (10). This virus isolate synthesized only a trace amount of mRNA 3-1 (10). The analysis of several other recombinant viruses with different genome structures suggested that mRNA 3-1 was synthesized only by viruses with a leader RNA containing three copies of UCUSA and gene 3 sequence derived from A59 but not JHM (10, 13). These observations suggested a new type of optional transcription initiation site, which was transcribed by the leader RNA with three UCUSA copies but not those with two copies, opposite to the transcription pattern of mRNA 2-1. We, therefore, attempted to further characterize this site. Figure 1 shows that mRNA 3-1 was synthesized in large quantity in cells infected with B1(3) but not with B1(2) viruses, which contained three and two copies of UCUSA, respectively. However, neither JHM(2) nor JHM(3) synthesized this mRNA, even though the latter contained three copies of UCUSA. Thus, other se-

quences, probably transcription initiation sequence, also accounted for the presence or absence of mRNA 3-1 transcription. In contrast, mRNA 2-1 was synthesized only by JHM(2) but not by B1(2) viruses, although both had two UCUSA copies. This was consistent with our previous conclusions (7, 10), since B1(2) virus had an A59-derived gene 2-1 sequence (12), which had a defective transcription initiation sequence for this gene (7, 11). Similar to the previous observation (7), JHM(2) synthesized a smaller amount of mRNA 2 than JHM(3). However, the relative amount of this mRNA was variable from virus preparation to preparation. An additional minor RNA species, 1-1, was noted in B1(3)- and JHM(3)- but not in B1(2)- or JHM(2)-infected cells, suggesting that it was also a differentially regulated transcription initiation site, which was transcribed by the leader RNA with three UCUSA copies, but not with two copies. Several of these viruses also had additional

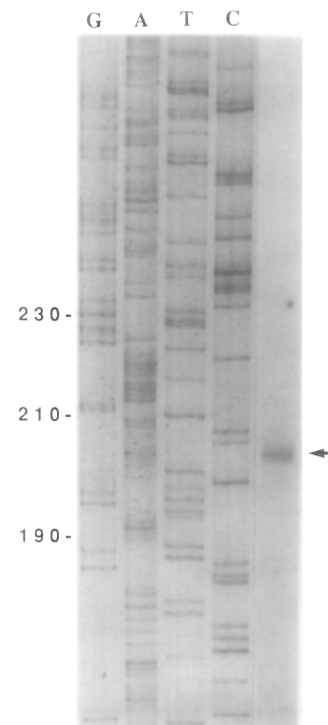


Fig. 2. Primer extension analysis of the 5'-end of mRNA 3-1 from B1(3). The synthetic oligonucleotide 348 was 5'-end-labeled with [γ - 32 P]ATP by polynucleotide kinase and extended with reverse transcriptase as previously described (9). Briefly, poly(A)⁺ RNA from B1(3)-infected DBT cells was denatured with 10 mM methylmercury and incubated at 45° for 30 min in 50 μ l reaction buffer containing 60 U of RNasin (Promega Biotech), 10 mM MgCl₂, 100 mM KCl, 50 mM Tris-hydrochloride, pH 8.3, 10 mM dithiothreitol, 12.5 mM each of dATP, dCTP, dGTP, TTP, and 35 U avian myeloblastosis virus reverse transcriptase (Seikagaku). Reaction products were analyzed by electrophoresis on a 6% polyacrylamide gel containing 8.3 M urea. A sequencing ladder was used as size markers. The primer extended product is indicated by an arrow.

TABLE 1

SEQUENCES OF TRANSCRIPTION INITIATION SITES OF VARIOUS mRNAs				
mRNA	Virus	Copy number of UCUAA in the leader	Intergenic site	mRNA synthesis
(A) 3-1	B1 (3)	3	UCUAAUCUAUC	+++
	B1 (2)	2	UCUAAUCUAUC	±
	A59	2	UCUAAUCUAUC	±
	A59 ^a	2	UCUGCUCUAUC	N.D. ^b
	JHM (2)	2	UCUGCUCUAUC	—
(B) 2-2	JHM (3)	3	UCCGGGUCUUGA	—
	MHV-1	3	UUUAA-UCUUGA	+++
		2	UUUAA-UCUUGA	±

Note. The genomic sequence at the intergenic site for mRNA 3-1 was obtained by direct sequencing of cDNA spanning this region, which had been amplified by PCR using oligomers 197 and 198 as specific primers. cDNA synthesis and PCR amplification were carried out as previously described (6). Oligomer 198 (5'-GTTC-CAAGCTTGACCACCGGTGCAATTA-3') was complementary to nucleotides 2676–2695 of gene 3, and oligomer 197 (5'-GACTCGAATTCAGTTGATAAGTTTGTGTACCCCG-3') was identical to nucleotides 1166–1191 of the same gene (14). The MHV-specific cDNA products representing nucleotides 1166–2695 of gene 3 were excised from low melting agarose (SeaPlaque, FMC Bioproducts), extracted with phenol/chloroform, and sequenced by dideoxyribonucleotide chain-termination method (16). Sequence determination for the transcription initiation site of mRNA 2-2 was described previously (6). Underscored letters indicate diverged sequences.

^a Sequence obtained from Luytjes *et al.* (14).

^b Not determined.

minor RNA species between mRNAs 3 and 4. As discussed previously (6), these RNAs most likely represented ribosomal RNAs, which could be removed by purification on an oligo(dT) column.

These results suggested further that mRNA transcription depends not only on the leader sequence but also on the transcription initiation sequence. Therefore, we determined the sequences of the transcription initiation sites for these optional RNAs. From the nested-set structure of MHV mRNAs and on the basis of the migration pattern of the RNAs, the transcriptional start site of mRNA 3-1 was predicted to be located within gene 3 of B1(3) virus at a site approximately 2 kb downstream from the initiation site of mRNA 3. To determine its exact location, we performed primer extension studies on poly(A)⁺ RNA from B1(3)-infected DBT cells. A synthetic oligonucleotide (oligo 348, 5'-ATTAGGAAGCGCCTCATCCG-3') complementary to nucleotides 2072–2092 of the published A59 gene 3 sequence (14) was used as a primer. The 5'-end-labeled primer was hybridized to poly(A)⁺ RNA and extended with reverse transcriptase. Primer extension products were then analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea (10).

As shown in Fig. 2, an extension product of 204 nucleotides was detected. Since the leader sequence of B1(3) was approximately 75 nucleotides long (8, 9), this result suggested that the transcription initiation site of mRNA 3-1 was located around nucleotide 1955 of gene 3. This conclusion was also supported by the primer extension reaction using a different primer, oligo 42 (5'-GATACCCAGTAGTTTCTG-3'), which was complementary to nucleotides 2151–2171 of gene 3 and was located 80 bases downstream of the oligo 348 binding site. The extension product was 284 nucleotides long (data not shown), consistent with the transcription initiation site determined using oligo 348 as a primer.

To precisely define the transcription initiation site of mRNA 3-1, the 5'-unique portion of mRNA 3-1 was cloned after amplification by polymerase chain reaction (PCR). For this purpose, intracellular RNA from B1(3)-infected cells was reverse transcribed using oligo 42 as a primer and then amplified with oligo 78 (5'-AGCTTTACGTACCCTCTCTACTCTAAACTCTT-TGTACTTT-3') as a second primer. The latter primer was homologous to the middle portion of the leader RNA and contained seven additional nucleotides representing the *Sna*BI site (15). The 280-nucleotide-long PCR product thus represented the 5'-end (including the leader sequence) of mRNA 3-1 and was cloned into the *Sma*I site of the pTZ18U vector (U.S. Biochemicals) by blunt-end ligation as previously described (6). Nucleotide sequence analysis of cDNA clones showed that the sequence of mRNA 3-1 diverged from that of the genomic RNA immediately upstream of the sequence UCUAAUCUAUC, which was located at nucleotides 1950–1961 of gene 3 (14), indicating that this region was the transcription initiation site of mRNA 3-1 (data not shown) (Table 1). Significantly, the copy number of UCUAA sequence in the leader region of different cDNA clones varied from two to four (data not shown). This observation was in agreement with the previously described heterogeneity of MHV mRNA, possibly resulting from the imprecise interaction between the UCUAA sequence at the 3'-end of the leader RNA and the transcription initiation sequence (9). This finding further established that this sequence represented a bona fide transcription initiation site of MHV mRNA.

However, the sequence determined here was different from the corresponding sequence of the published A59 gene 3 (14) (Table 1), even though B1(3) virus derived its gene 3 from the parental A59 virus (12). This result suggested that the parental A59 virus used in our laboratory may have a different sequence in this region. We therefore used the same procedures to sequence the A59 used in our laboratory. The results showed that its sequence was identical to that of B1(3)

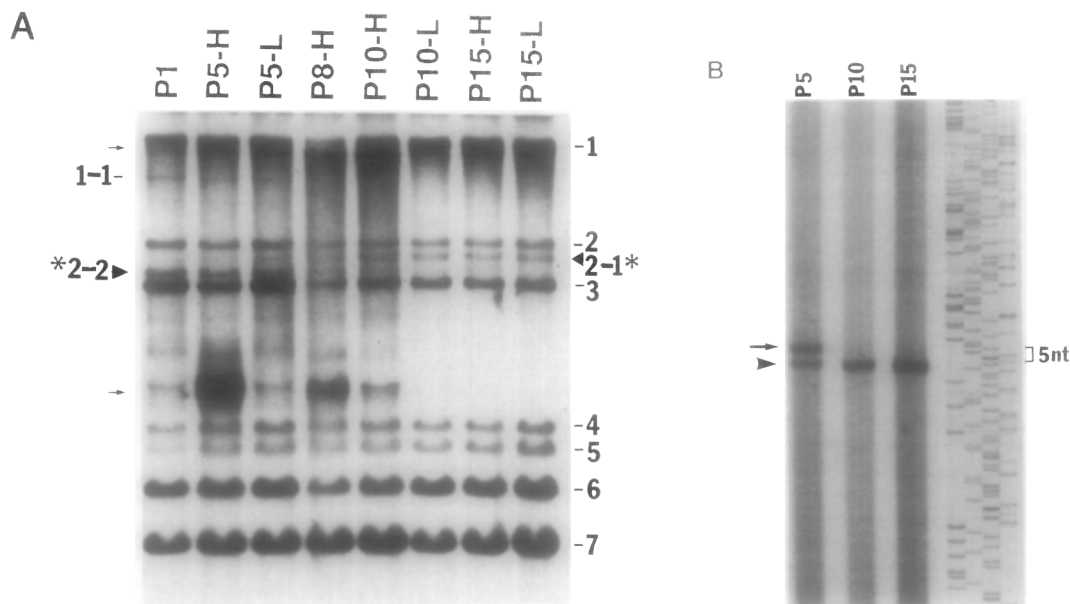


Fig. 3. Alteration of mRNA synthesis and leader RNA sequence of MHV-1 during serial passage in DBT cells. (A) MHV-1 was plaque-purified twice and propagated twice on DBT cells to generate a high-titer virus stock (designated P1). Subsequently, 0.2 ml of the virus stock was added onto a fresh 6-cm plate of DBT cells, and the supernatant (designated P2) from this culture was collected after 24 hr. Thirteen subsequent passages were performed by the same procedure (designated P2-H to P15-H). DBT cells infected with supernatants P5-H, P8-H, P10-H, and P15-H were used for ^{32}P -labeling of intracellular viral RNA. To eliminate defective-interfering (DI) RNA, virus stocks from each passage were diluted 10^4 - to 10^5 -fold and used to infect DBT cells at a multiplicity of infection (m.o.i.) of 0.0001. The supernatant was harvested 24 to 36 hr p.i. Some of the supernatants from low m.o.i. infection (P5-L, P10-L, and P15-L) were used to infect fresh DBT cells and labeled with [^{32}P]-orthophosphate. RNAs were analyzed by electrophoresis as described in Fig. 1. mRNA 2-1 and 2-2 are indicated by asterisks. The DI RNAs are indicated by arrows. (B) The virion RNA of P5-H, P10-H, or P15-H virus was used for primer extension studies using a primer complementary to nucleotides 172–188 from the 5'-end of JHM genomic RNA according to the procedures as described in Fig. 2. As demonstrated previously (9), the extended product was 188 nucleotides long if the leader RNA contained three UCUAA copies (indicated by an arrow) and 183 nucleotides long if it contained two copies (indicated by an arrowhead). A DNA sequencing ladder was run in parallel to serve as size markers.

virus in this region, confirming that this was the authentic sequence of the parental and recombinant viruses (Table 1). This result also explained why mRNA 3-1 was not synthesized by JHM(3), inasmuch as the sequence of JHM in this region was the same as that of the published A59, which did not make mRNA 3-1 (Table 1). Additionally, we sequenced B1(2) virus, which failed to make mRNA 3-1, and found that its sequence was identical to that of B1(3) and the parental A59 within this region. The sequence identity between B1(2) and B1(3) extended for at least 100 nucleotides on either side of the transcription initiation site for mRNA 3-1. Therefore, we concluded that the difference in mRNA 3-1 synthesis by B1(2), B1(3), and A59 was most likely due to the difference in the copy number of the UCUAA pentanucleotide in the leader RNA.

We have recently identified another novel mRNA species (mRNA 2-2) in cells infected with another strain of MHV, MHV-1 (6). The transcription initiation sequence of this mRNA was similar to but slightly different from the consensus sequence for MHV mRNAs (Table 1). To determine whether this novel RNA was

differentially regulated by the copy number of UCUAA in the leader RNA, we performed undiluted serial passages of MHV-1 in DBT cells. The ^{32}P -labeled intracellular viral RNA isolated from virus-infected cells at different passage levels were examined by electrophoresis on agarose gel. To rule out the effects of defective-interfering (DI) RNA, we have also examined intracellular viral RNAs after a low multiplicity of infection (m.o.i.) passage of the different virus isolates to remove DI RNAs. Figure 3A shows that the original MHV-1 synthesized mRNA 2-2 in addition to the seven standard mRNAs. After 10 passages in DBT cells, the virus did not synthesize mRNA 2-2, but instead synthesized mRNA 2-1, which had previously been characterized as being transcribed by the leader RNA with two UCUAA copies (7). At both passages 5 and 8, both of these mRNAs were synthesized. No difference in the intracellular viral RNA pattern could be detected in the presence or absence of DI RNA at every passage level. To determine whether the change of mRNA 2-1 and 2-2 expression correlated with the UCUAA copy number in the leader RNA, we examined the structure of

TABLE 2
MINOR TRANSCRIPTION INITIATION SITES WITHIN GENE 1

Leader 5'	41 ---AAACTCTTGTAGTTTAAATCTAATCTAATCTAAAC			
	Primer 78	1	2	3
Genome	5780 T T T A A T G T A C C A T T T T A A T C T G C T C C - - -			
mRNA	T A G T T T A A A T C T A A T C T A A T C T (G C T C C) - - -	1	2	3
Genome	5888 C A T G T G A A A T G T A A A C C C A A - - -			
mRNA	T A G T T T A A A T C T A A A · (C C C A A) - - -	1		
Genome	7923 A G T T G A A A C G C C C T G T G A A T C C A A C A G A - - -			
mRNA	T A G T T T A A A T C T A A T C T A A T C T A A (C A G A) - - -	1	2	3

Note. Oligonucleotide primers complementary to different regions of gene 1 (around nucleotides 5942 and 7999, respectively) (2) were used separately as primers for reverse transcription of the intracellular RNA from JHM(3)-infected cells. A second primer (78) representing leader RNA sequence (nucleotides 22 to 56) was then used for PCR amplification by *Taq* polymerase. The PCR products were cloned into pTZ18U vector and sequenced. The sequences were aligned with the published gene 1 sequence (2). The regions where the mRNA sequences diverged from the published genomic sequences were identified, which represented the transcription initiation sites for these mRNAs. The UCUAA copy numbers within the leader RNA of each mRNA are indicated. The nucleotide numbers indicated in the genomic sequences represent nucleotide positions from the 5'-end of the genome (2).

the leader RNA of MHV-1 at different passage levels by primer extension and sequencing. The results showed that both virus isolates from passage levels 10 and 15 had two UCUAA copies (Fig. 3B), in contrast to the original MHV-1, which had three UCUAA copies (6). Interestingly, the virus at passage level 5 contained a mixture of two and three UCUAA copies, corresponding to the detection of both mRNA 2-1 and 2-2 (Fig. 3A). This result suggested that the leader sequence of MHV-1 underwent evolution from three to two UCUAA copies during passage in culture, similar to that of JHM virus (10), and that the synthesis of mRNAs 2-2 and 2-1 correlated well with the presence of three and two UCUAA copies in the leader, respectively. The entire MHV-1 leader sequence was identical to that of JHM (data not shown). This result indicated that the initiation site of mRNA 2-2 of MHV-1 was utilized by the leader sequence with three but not two UCUAA copies. Additionally, RNA 1-1 was also found to be transcribed only by MHV-1 with three UCUAA copies, but not by those with two copies (Fig. 3A), consistent with the findings with JHM and B1 viruses (Fig. 1).

We attempted to determine the transcription initiation site of RNA 1-1 by PCR cloning. Using various primers representing sequences of different regions of

gene 1 of the JHM strain of MHV (2) and a second primer representing the 5'-end of the leader RNA, we detected several PCR products which represented mRNAs initiated from various sites. These transcription initiation sequences appeared to bear some resemblance to the consensus transcription initiation signal (Table 2). We have also attempted to perform primer extension studies to determine which of these was the transcription initiation signal for RNA 1-1. However, since the transcription initiation site of RNA 1-1 was located within a gene of 22 kb (2) and the molar amount of this RNA was much less abundant than the other mRNAs, the results were not conclusive. Nevertheless, the detection of these PCR products with the correct leader sequence indicated that these sequences, which were similar to the consensus intergenic sequence, could be utilized as transcription initiation sites, although at a very low efficiency.

To determine whether these optional and differentially regulated RNAs encode any truncated products of the viral proteins, we examined the virus-specific protein synthesis in the cells infected with B1(3) and other viruses by labeling with [³⁵S]methionine and precipitating with antiserum specific for MHV. No proteins other than the S, N, M and, in some cases, HE, were

detected (data not shown). Thus, mRNAs 2-2 and 3-1 are most likely not functional. However, other possibilities, such as rapid turnover of the protein or the inefficiency of immunoprecipitation because of the change of epitopes on the protein, cannot be ruled out. Since the gene product of mRNA 1 is a nonstructural protein and has not been identified, we cannot determine whether mRNA 1-1, which represents a truncated version of mRNA 1, is functional.

These studies have identified, thus far, at least four transcription initiation sites which were regulated in a different manner by the leader RNA with different copy numbers of UCUAA sequence. Several of these (mRNAs 2-2, 3-1, and 1-1) were transcribed efficiently by the leader sequence with three UCUAA copies, while the other (mRNA 2-1) was transcribed efficiently by those with two copies. It is not clear how the interaction of these intergenic sequences with the leader RNA containing different copy numbers of UCUAA can affect transcription initiation. It is possible that this interaction requires RNA-protein interactions involving RNA polymerase and transcription factors, but not simply base-pairing between the leader RNA and the intergenic sequences. The three-dimensional structure of RNA may be affected by the copy number of UCUAA. Since these intergenic sequences are identical in the same pair of viruses studied here, these results strongly suggest that the leader RNA plays a regulatory role for transcription. This interpretation is most consistent with the leader-primed transcription mechanism (7) proposed for MHV mRNA synthesis.

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