Coronavirus Leader RNA Regulates and Initiates Subgenomic mRNA Transcription both in *trans* and in *cis*

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Mouse hepatitis virus (MHV), a coronavirus, utilizes a discontinuous transcription mechanism for subgenomic mRNA synthesis. Previous studies (C.-L. Liao and M. C. C. Lai, *J. Virol.* 68:4727–4737, 1994) have demonstrated that an upstream *cis*-acting leader sequence serves as a transcriptional enhancer, but the mechanism of transcriptional regulation is not clear. In this study, we constructed a series of defective interfering (DI) RNAs containing the chloramphenicol acetyltransferase (CAT) gene behind a differentially expressed transcription initiation (intergenic) sequence (for mRNA2-1). These DI RNAs had different copy numbers of the UCUAA pentanucleotide sequence at the 3' end of the leader. Transfection of these DI RNA constructs into cells infected with a helper MHV, which contains either two or three UCUAA copies at the 3' end of the leader, resulted in differential expression of CAT activities. We demonstrated that the copy number of UCUAA repeats in the leaders of both helper viral and DI RNAs affected the level of CAT activity, suggesting that MHV leader RNA could regulate both in *trans* and in *cis* the transcription of subgenomic mRNAs. The leader RNA of subgenomic mRNAs was derived from either the *trans*- or the *cis*-acting leader. Furthermore, insertion of a UA-rich sequence (UUUAUAAAC) immediately downstream of the leader in DI RNA, to match the sequence of helper viral RNA, enhanced the CAT activity by threefold, suggesting that this nine-nucleotide sequence is a *cis*-acting element. Interestingly, when the nine-nucleotide sequence was absent in DI RNA, the leaders of subgenomic mRNAs were exclusively derived from the helper virus. In contrast, when the nine-nucleotide sequence was present in DI RNA, the leaders were derived from both helper viral and DI RNAs. These results suggest that the nine-nucleotide sequence either is required for the leader RNA to initiate mRNA synthesis or, alternatively, serves as a transcription terminator for the leader RNA synthesis. However, when a constitutively expressed intergenic sequence (for mRNA7) was used, no difference in transcription efficiency was noted, regardless of the copy number of UCUAA in the DI RNA and helper virus. This study thus indicates that MHV subgenomic RNA transcription requires the interaction among the intergenic (promoter) sequence, a *trans*-acting leader, and a *cis*-acting leader sequence. A novel model of transcriptional regulation of coronavirus subgenomic mRNAs is presented.

Murine coronavirus mouse hepatitis virus (MHV) contains a single-stranded, positive-sense RNA genome of approximately 31 kb (7, 13). In MHV-infected cells, six to seven species of virus-specific subgenomic mRNAs, which are conested on the 3' end of the genome, are synthesized (9, 14). All of the mRNAs contain identical leader sequences of 72 to 77 nucleotides, which are derived from the 5' end of the genomic RNA (8, 11, 31). A considerable body of evidence which is consistent with a leader-primed transcription mechanism for MHV subgenomic mRNAs has been obtained (reference 7 and references herein). This model proposes that a leader RNA is transcribed from the 3' end of the genome-size, negative-strand template RNA, dissociates, and then rejoins the same or different template RNA at downstream intergenic regions to serve as the primer for mRNA transcription. The critical sequences in this transcription mechanism include the 3' region of the leader sequence, which consists of two to four copies of a pentanucleotide sequence (UCUAA) (21, 22), and the intergenic sequences (ISs) preceding each viral gene, which include a consensus UCUAAAC or similar sequence (7, 30). The fusion of the leader sequence with the mRNA body sequence occurs between the UCUAA repeats of the leader and the consensus IS (9, 12, 30, 31). Mutations of the ISs affect

the efficiency of mRNA transcription (6, 19). These findings suggest that MHV mRNA synthesis requires both the leader RNA and the ISs.

It has been shown that the leader RNA of mRNAs can be switched readily between two different MHVs during coinfection, suggesting that leader RNA and mRNAs are in two separate transcription units (24). Furthermore, in an *in vitro* transcription system, an exogenously added leader RNA can be incorporated into mRNAs, suggesting an *in trans* initiation of mRNA synthesis (2). These results are consistent with the leader-primed transcription model (7). However, this transcription model does not explain the detection of subgenomic negative-strand RNAs (28) and subgenomic replicative-form RNAs (27) in coronavirus-infected cells. Also, because of the potential occurrence of RNA recombination in coronaviruses (20), the detection of leader RNA switching during MHV coinfections could be interpreted as the result of RNA recombination. Thus, an alternative model of discontinuous transcription for coronavirus has been proposed; i.e., the discontinuous transcription step occurs during negative-strand RNA synthesis, and subgenomic mRNAs are transcribed faithfully from these subgenomic negative-strand RNA templates (27).

One critical, but not yet established, component of the leader-primed transcription model is that the leader RNA can regulate and prime in *trans* subgenomic mRNA synthesis. The finding that different MHVs which contain either two or three copies of the UCUAA pentanucleotide sequence in the leader

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FIG. 1. Structures and sequences of the cDNA clones used. (A) Structure of plasmid p25CAT and its derivatives, DECAT2-1, DECAT2-1(3), and DECAT2-1(2). The restriction enzyme sites used for cloning and construction are indicated. Each unlabeled narrow box in the leader and the IS represents a copy of UC₃UAA. The arrow indicates the transcription initiation site of subgenomic RNA. The V-shaped breaks in the lines represent a nine-nucleotide deletion. The total lengths of the constructs are drawn approximately to scale. Only the DI-derived regions are shown for DECAT2-1, DECAT2-1(3), and DECAT2-1(2). (B) Comparison of the inserted ISs in 25CAT and DECAT2-1. The consensus sequences are underlined. The ISs derived from viral RNA are marked at the top. The eight nucleotides between the *SpeI* site and the IS in 25CAT were derived from pBluescript. ORF, open reading frame. (C) Sequences of the leader regions of viral RNAs and DI RNA constructs used. The identical sequences are represented by solid lines. The pentanucleotide repeats are underlined, and the nine-nucleotide sequences immediately downstream of the repeats are underlined with double lines or dashes, if deleted. Each number at the top indicates the nucleotide position from the 5' end.

RNA transcribe different amounts of mRNA2-1 (21, 29, 32) gave the first indication that the MHV leader RNA regulates and most likely initiates viral mRNA transcription. Similar transcriptional regulation has also been noted for several other recently detected mRNAs, including mRNA2-2 and mRNA3-1 (12). However, in natural MHV infections, the leader RNA and the transcription initiation site of every mRNA are in the same genomic RNA molecule; thus, the most critical question of whether leader RNA regulates transcription *in trans* remains unanswered.

We have recently described a defective interfering (DI) RNA-reporter system which includes a chloramphenicol acetyltransferase (CAT) gene behind an IS, thus enabling the detection of subgenomic mRNA transcription by measuring the CAT activity (16). By using this system, we have demonstrated that the leader RNA and additional 5'-end genomic sequences are required as an upstream *cis*-acting element for subgenomic mRNA transcription. The IS used in that study (16) was a constitutively expressed sequence from MHV mRNA7; thus, its usefulness for studying the mechanism of transcriptional regulation is limited. In this report, we have further modified this system by using an IS of a differentially expressed mRNA (mRNA2-1) instead of constitutive mRNA7. We demonstrate that MHV mRNA transcription is regulated by the leader RNA *in trans* and that the *trans*-acting leader RNA is incorporated into subgenomic mRNAs. Furthermore, the leader RNA also regulates mRNA synthesis *in cis*. Thus, coronavirus RNA transcription involves the interaction of three components: the *cis*-acting leader sequence at the 5' end of the genomic RNA, the transcription initiation (promoter) sequence, and a leader sequence *in trans*. This study thus establishes a unique mechanism of transcriptional regulation by an RNA *in trans*.

MATERIALS AND METHODS

Viruses and cells. MHV strains JHM(2), JHM(3) (21), MHV-1(2), and MHV-1(3) (32) were used throughout this study. JHM(3) and MHV-1(3) contain three UC₃UAA repeats at the 3' end of the leader RNA. JHM(2) and MHV-1(2) contain two UC₃UAA repeats and were derived from JHM(3) and MHV-1(3), respectively, after 15 passages of undiluted virus preparations in cell culture. The properties of these viruses have been described previously (21, 32). DBT cells (4) were used for infections and transfections.

Plasmid construction. Standard procedures (1, 25) were used throughout.

p25CAT. Plasmid p25CAT was constructed as described previously (16). It contains the sequence of DE25 (22), the CAT gene, and the IS between genes 6 and 7 (IS7) of MHV strain A59.

pDECAT2-1. Plasmid p25CAT was linearized with *SpeI* and subjected to PCR amplification. The primer pair 3'CAT542 and 5'Spe2-1CAT was used in PCR. Primer 5'Spe2-1CAT contains an *SpeI* site at the 5' end, the IS between genes 2 and 2-1 (IS2-1) of MHV strain JHM, and the first 15 nucleotides of the CAT gene (5'-TAACTAGTAGTGTAATAAACTTAT TATTTTGTGAAATGGAGAAAAAAT-3'). The resulting 0.7-kb PCR fragment was digested with *SpeI* and *BspEI*, and the 0.2-kb *SpeI*-*BspEI* fragment was cloned into the unique *SpeI* and *BspEI* sites of p25CAT. Thus, pDECAT2-1 differs from p25CAT only in the IS (between *SpeI* site and the CAT open reading frame) (Fig. 1B).

pDECAT2-1(2) and pDECAT2-1(3). The 5'-end 0.5-kb segment, including the leader sequence, of pDECAT2-1 was replaced with the corresponding sequence of wild-type JHM RNA by the following strategy. The 5'-end 0.5-kb cDNA fragment of the JHM(2) or JHM(3) viral genome was gener-

ated by reverse transcription (RT) of the purified viral genomic RNA, using primer 3'-499 (5'-CATCATAGTCGAGGCCTC CAC-3', complementary to the nucleotides 470 to 499 of gene 1, including an endogenous *StuI* sequence) (13) and PCR using primers 3'-499 and 5'L9 (5'-TGATTGGCGTCCGTACG TACC-3', corresponding to nucleotides 9 to 28 of the leader RNA, including an endogenous *SnaBI* site). The PCR fragment was digested with *StuI* and *SnaBI* and used to replace the *SnaBI-StuI* fragment of pDECAT2-1. The resulting constructs containing the *SnaBI-StuI* fragment derived from JHM(2) and JHM(3) are designated pDECAT2-1(2) and pDECAT2-1(3), respectively.

pDECAT2-1(2)m and pDECAT2-1(3)m. The leader sequences of pDECAT2-1(2) and pDECAT2-1(3) were replaced with the mutated leader sequence between the *SnaBI* and *StuI* sites. Using an altered 5' primer (5'L17m; 5'-GTCCGTACG TACCTAATCTACTCTAA-3') and 3'A499 as the 3' primer, a mutated PCR fragment was generated from DECAT2-1(2) or DECAT2-1(3) as the template and cloned back into the *SnaBI* and *StuI* sites of DECAT2-1(2) or DECAT2-1(3). The resulting constructs, pDECAT2-1(2)m and pDECAT2-1(3)m, differ from their parental constructs by three nucleotides (CUC to UAA) (Fig. 1C).

p25CAT+9nt and pDECAT2-1+9nt. p25CAT+9nt and pDECAT2-1+9nt differ from p25CAT and pDECAT2-1 in that they contain the nine-nucleotide sequence (UUUAUAAAC) immediately downstream of the UCUA repeats in the leader. To insert the nine nucleotides, several sets of PCR were performed. In the first PCR, a universal T7 promoter -40 primer (corresponding to the sequence of pBluescript-SK+ upstream T7 promoter) and the primer 3'-4rep9nt (5'-GAAGTGCCGTTTATAAAGTTTAGATTAGATTAGAT TAGA-3', complementary to the sequence surrounding the junction site between leader and the genome with nine-nucleotide insertion) were used. In the second PCR, primers 3'-499 and 5'-4rep9nt (5'-TCTAATCTAACTTTATAAAG GGCATTTCCTGCG, corresponding to the sequence surrounding the junction site between leader and the rest of the genome with nine-nucleotide insertion) were used. p25CAT DNA was used as the template for both sets of PCR. In the third PCR, a small portion of the first two PCR products was denatured and reannealed to serve as a template, and the universal -40 primer and 3'-499 were used as the primers. PCR products from the last set were digested with *SnaBI* and *StuI*, and the 0.5-kb *SnaBI-StuI* fragment was cloned into the large *SnaBI-StuI* fragments of p25CAT and pDECAT2-1 to substitute the small *SnaBI-StuI* fragments, generating p25CAT+9nt and pDECAT2-1+9nt, respectively.

RNA transcription and transfection. All plasmid constructs were linearized with *XbaI* and transcribed in vitro with T7 RNA polymerase as recommended by the manufacturer (Promega). RNA transfection was carried out by the DOTAP method (Boehringer Mannheim). Briefly, DBT cells (in a 60-mm-diameter petri dish) were infected with a helper virus at a multiplicity of infection of 5. At 1 h postinfection, a mixture of 20 μ l of RNA (5 μ g) and 10 μ l of DOTAP in BHS buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4], 150 mM NaCl) was added to the culture medium (2 ml), and cells were incubated for various lengths of time as indicated.

CAT assay. Infected and transfected DBT cells from a 60-mm-diameter petri dish were harvested at 8 h postinfection in most experiments, resuspended in 150 μ l of 0.25 M Tris-HCl (pH 8.0), and lysed by freezing and thawing three times. Cellular lysates were incubated at 60°C for 10 min, and 50 μ l of each sample was assayed for CAT activity, using an assay kit

TABLE 1. Analysis of *trans* and *cis* activations of CAT expression by the leaders of viruses and DI RNA derivatives

Transfected RNA	CAT activity ^a with:	
	JHM(3)	JHM(2)
25CAT	291 $\pm$ 7.6	306 $\pm$ 2.8
DECAT2-1	4 $\pm$ 1.6	37 $\pm$ 8.6
DECAT2-1(3)	22 $\pm$ 9.3	260 $\pm$ 43.4
DECAT2-1(2)	70 $\pm$ 17.5	270 $\pm$ 27.2

^a Expressed is indicated as fold increase over the background value for a culture in which virus infection alone was used as a negative control, which was set at onefold. Values are means and standard deviations of five separate infection and transfection experiments in DBT cells.

as recommended by the manufacturer (Promega). The CAT reaction was carried out for 12 h.

RT-PCR and cloning of subgenomic mRNAs. Total RNAs were isolated from MHV-infected and RNA-transfected DBT cells at 8 h postinfection by the Nonidet P-40 method (17) and used for cDNA synthesis by RT, using the CAT-specific primer 3'CAT542. An additional primer specific to the leader was used for the subsequent PCR amplification. The 0.7-kb PCR fragment, which represents the 5' portion of the subgenomic CAT-containing RNA, was digested with *SnaBI* and *EcoRI*, and the *SnaBI-EcoRI* fragment (0.2 kb) was cloned into the *SmaI* and *EcoRI* sites of pBluescript.

DNA sequencing. The sequences of all constructs and the leader and leader-body fusion sites of all cDNA clones were determined by dideoxynucleotide chain termination sequencing (26). Primers 3'-A140 (5'-AATGTCAGCACTATGA CAAG-3') and 3'-CAT106 (5'-TCTGGTTATAGGTACAT TGA-3') were used to determine the sequences of genomic and subgenomic RNA leaders, respectively.

RESULTS

The leader sequence of helper virus *trans* regulates the expression of CAT activity. Previously, we used a DI RNA containing the CAT gene placed behind the IS of mRNA7 (termed p25CAT) (Fig. 1A) to determine the upstream sequence requirement for MHV mRNA synthesis (16). When the in vitro-transcribed RNA is transfected into MHV-infected cells, the CAT activity reflects the efficiency of subgenomic mRNA transcription from the IS (16). However, because mRNA7 is a constitutively expressed mRNA, the IS for this mRNA is not useful for studying the mechanism of transcriptional regulation. We therefore constructed a new DI vector containing the IS of the differentially transcribed mRNA2-1. We have previously shown that mRNA2-1 is transcribed by MHVs containing two UCUA repeats in the leader sequence but not by viruses with three repeats (29, 32); however, it is not clear whether the leader sequence exerts this transcriptional regulation in *trans* or in *cis*. To address this question, we made a construct, DECAT2-1 (Fig. 1A), which contains the IS, including the core sequence (UAAUAAAC), of mRNA2-1 (in contrast to UCUAUCUAAAC for mRNA7) (Fig. 1B). The DECAT2-1 RNA was transfected into cells that had been infected with either JHM(2) or JHM(3) virus, containing two or three copies, respectively, of the UCUA sequence (21). The results showed that in JHM(2)-infected cells, CAT activity increased by 37-fold, while in JHM(3)-infected cells, it increased by only 4-fold (Table 1). In contrast, a high level of CAT activity was expressed from 25CAT RNA in both JHM(2)- and JHM(3)-infected cells (about 300-fold increase) (Table 1). These results indicate that subgenomic RNA was

TABLE 2. Origin and number of the pentanucleotide repeats of subgenomic DI RNA leader

Group	Transfected RNA	JHM(2)-infected cells			JHM(3)-infected cells		
		Origin of the leader (no. of clones/no. tested)		No. of UCUAA repeats	Origin of the leader (no. of clones/no. tested)		No. of UCUAA repeats
		Helper virus	DI RNA		Helper virus	DI RNA	
I	25CAT	12/12	0/12	2	12/12	0/12	3
	DECAT2-1	12/12	0/12	2	12/12	0/12	3
II	DECAT2-1(2)m	10/12	2/12	2	11/12	1/12	2 and 3
	DECAT2-1(3)m	11/12	1/12	2 and 3	9/12	3/12	3
III	DECAT2-1(2)	NK ^a	NK	2	NK	NK	2 and 3
	DECAT2-1(3)	NK	NK	2 and 3	NK	NK	3
IV	25CAT+9nt	11/16	5/16	2 and 4	10/16	6/16	3 and 4
	DECAT2-1+9nt	12/16	4/16	2 and 4	11/16	5/16	3 and 4

^a NK, not known.

differentially transcribed from the IS of mRNA2-1, depending on the number of UCUAA repeats in the leader sequence of the helper virus, whereas subgenomic RNA was constitutively transcribed from the IS of mRNA7 regardless of the leader sequence of the helper virus. These results mimic the natural transcriptional patterns of JHM(2) and JHM(3) (21) and demonstrate that the leader RNA of a helper virus regulates *in trans* subgenomic mRNA transcription. Similar results of differential transcription were obtained when two isolates of different MHV strains, MHV-1(2) and MHV-1(3), which have two and three UCUAA copies, respectively (32), were used as helper viruses (data not shown), confirming that the transcription efficiency of the mRNA2-1 IS is regulated by a *trans*-acting leader sequence. The CAT expression from DECAT2-1 was approximately 8-fold lower than that from 25CAT when JHM(2) was used as a helper virus (Table 1), indicating that the IS of mRNA2-1 is not as efficient as that of mRNA7. These results suggest that the efficiency of subgenomic mRNA transcription is regulated by both the IS sequence and the leader sequence of the helper virus *in trans*.

The leader sequence of DI RNA also affects the expression of CAT activity. Since the leader sequence is present not only in the helper virus RNA but also in the DI RNA, we next examined whether the leader sequence of DI RNAs could also regulate mRNA transcription. This effect will be *cis* acting, since the leader RNA and IS are in the same molecule. We replaced the leader of DECAT2-1 with JHM viral leader RNA containing either two or three copies of the pentanucleotide sequence, yielding DECAT2-1(2) and DECAT2-1(3), respectively (Fig. 1A). The JHM leader RNA differs from that of DI RNA (as represented in p25CAT) in four nucleotides and copy number of UCUAA and in the presence of an additional nine-nucleotide sequence immediately downstream (Fig. 1C). As shown in Table 1, both DECAT2-1(3) and DECAT2-1(2) yielded almost the same level of CAT activity as 25CAT in JHM(2)-infected cells, which was eight times higher than the level for DECAT2-1. In JHM(3)-infected cells, these two RNAs showed 22- and 70-fold increases, respectively, in CAT activity, which were significantly higher than that of DECAT2-1. These comparisons further showed that DECAT2-1(2) yielded a higher level of CAT activity than DECAT2-1(3), similar to the effects of helper viruses on DECAT2-1 expression. Since the only difference among these constructs is the structure of the leader RNA, these results indicate that the leader sequence in DI RNA can also regulate *cis* the transcription efficiency of subgenomic mRNA.

To rule out the possibility that the difference in CAT activity

observed between DECAT2-1(3) and DECAT2-1(2) was due to a difference in their replication efficiencies, we directly examined the amounts of these two DI RNA species in the cells by Northern (RNA) blot analysis using a CAT-specific probe. No difference in the amount of full-length DI RNA between DECAT2-1(2) and DECAT2-1(3) in transfected cells was detected (data not shown), suggesting that the observed difference in CAT activities between DECAT2-1(2) and DECAT2-1(3) was not due to the difference in their replicative efficiencies but to the difference in their transcriptional efficiencies.

Helper viruses provide the leader in *trans* to initiate subgenomic RNA transcription. We next examined the origin of the leader RNA in the CAT-containing subgenomic DI RNA, i.e., whether it was contributed by the helper virus *in trans* or DI RNA *in cis*. Cells were infected with JHM(2) or JHM(3) and transfected with 25CAT or DECAT2-1. Intracellular RNAs were isolated, and cDNAs were synthesized by RT with a CAT gene-specific antisense primer and amplified by PCR with a leader RNA-specific sense primer (corresponding to nucleotides 9 to 28) which does not discriminate the leaders of different origins (see Materials and Methods). The PCR products were cloned and sequenced. Since the leader sequences of the helper virus and DI RNAs differ by three nucleotides (CUC versus UAA, besides the difference in the copy number of the UCUAA sequence), which can be detected in the PCR products (Fig. 1C), the origin of the leader RNA in the subgenomic mRNAs could be determined with certainty.

Twelve clones of each group were sequenced. The results showed that all of the CAT-containing subgenomic mRNAs, irrespective of helper viruses or DI RNAs used in the studies, had the leader sequence derived exclusively from the helper virus, which contains CUC at nucleotides 30 to 32 (Table 2, group I; Fig. 2A). None of the clones contained a DI-derived leader sequence. The fusion between the leader and the body of subgenomic DI RNA took place between the UCUAA of the helper virus leader RNA and the UCUAAAC of the IS (Fig. 2A). The number of UCUAA repeats in the leader of the subgenomic RNA corresponded to those of helper viruses used (Table 2, Fig. 2A, and data not shown). These results established unequivocally that the helper virus provided the leader *in trans* to initiate subgenomic mRNA transcription, consistent with the leader-primed transcription mechanism. This result is particularly striking considering the finding that the leader sequence of DI RNA regulates *cis* mRNA transcription (Table 1), and yet it is not incorporated into subgenomic mRNA. Thus, we conclude that the leader RNA provides both

FIG. 2. Autoradiograms of sequencing gels showing the leader and the initiation site (fusion site) of the CAT-containing subgenomic RNAs. Each sequence was obtained from a single cloned PCR product and determined by the dideoxynucleotide chain termination method (26). Sequences are presented as RNA sequences. The UCUAA repeats are numbered. The triple nucleotides which are characteristic of the leader of the virus (CUC [A]) and DI RNA (UAA [B and C]) are indicated. The sequence of fusion site between the leader RNA and subgenomic mRNA body is shown. (A) mRNA from DECAT2-1-transfected and JHM(2)-infected cells. (B) mRNA from DECAT2-1(3)m-transfected and JHM(2)-infected cells. (C) mRNA from DECAT2-1(2)m-transfected and JHM(2)-infected cells. CAT-ORF, CAT open reading frame.

the regulatory and initiating functions for subgenomic mRNA synthesis and that these two functions are separable. This result further supports the conclusion reached in the accompanying report (16).

DI RNA could also contribute the leader in *cis* to subgenomic mRNAs. Since the leader RNA of subgenomic mRNAs is derived from the leader sequence of the same genomic RNA molecule during wild-type MHV mRNA transcription, we examined the conditions which will allow the leader RNA to be utilized in *cis* for mRNA initiation. We made two additional RNA constructs, DECAT2-1(2)m and DECAT2-1(3)m, which contain a leader sequence identical to that of the DI RNAs but contain two and three UCUAA repeats, respectively, and nine additional nucleotides (UUUA UAAAC) immediately downstream. This nine-nucleotide sequence resembles the consensus (UCUAAAC) sequence (30), but it is not a part of the leader sequence. It is deleted in most of the naturally occurring DI RNAs (10) (Fig. 1C). The two RNAs were transfected into JHM(2)- or JHM(3)-infected DBT cells, and the CAT-containing subgenomic mRNAs were amplified by RT-PCR and sequenced. The results showed that the leader sequences in 9 to 11 of the 12 PCR clones in all cases were derived from the helper viral RNAs (Table 2, group II; Fig. 2A). However, in contrast to 25CAT and DECAT2-1, 1 to 3 of the 12 clones contained the DI-derived leader sequence (Table 2; Fig. 2B and C). The fusion between the

leader and the body of subgenomic DI RNA took place between the last pentanucleotide repeat of the leader RNA and the consensus IS (Fig. 2B and C and data not shown).

We have also examined the leader sequences of the subgenomic RNA generated from DECAT2-1(2) and DECAT2-1(3). Since the leader sequences are identical to that of the helper viruses (except for an A at nucleotide 12, which could not be sequenced because of the limitation of the primer used), the origins of the leaders in the mRNA of these constructs could not be unequivocally determined. Nevertheless, in all cases, the number of the UCUAA repeats in the leaders of subgenomic RNAs corresponded to the combinations of the leaders from both helper viruses and DI RNAs used (Table 2, groups II and III, and data not shown), suggesting that both helper virus and DI RNA contributed the leader sequence to subgenomic mRNAs. This finding suggests that depending on the nature of the leader RNA sequence, the leader RNA could initiate in *cis* mRNA transcription and be incorporated into mRNAs.

Insertion of the nine-nucleotide sequence enhances the expression of CAT activity and promotes the utilization of the leader RNA for mRNA initiation. The foregoing results for DECAT2-1(3)m and DECAT2-1(2)m, both of which contain nine additional nucleotides downstream of the leader, suggest that the nine-nucleotide sequence may influence the *cis*-acting activity of the leader RNA. However, these two constructs

FIG. 3. Effects of the nine-nucleotide insertion in DI RNA on the expression of CAT activity. The scale indicates fold increase of CAT activity against the background, which is set at onefold (see the footnote to Table 1). Values are means and standard deviations from five independent experiments. JHM(2) or JHM(3) was used as a helper virus.

contain two and three UCUAA repeats, in contrast to the original DI RNA (DECAT2-1 and 25CAT), which has four UCUAA repeats (11). Therefore, to establish unequivocally the contributions of the nine-nucleotide sequence to the activity of leader RNA, we inserted the nine-nucleotide into DECAT2-1 to generate DECAT2-1+9nt (Fig. 1C). Transfection of DECAT2-1+9nt into JHM(2)- or JHM(3)-infected DBT cells resulted in an increase of CAT activity, which was three times higher than that induced by DECAT2-1, in both cells (Fig. 3). This difference was reproducible in five independent experiments. Furthermore, approximately one-third of the subgenomic mRNAs of DECAT2-1+9nt contained a DI (*cis*)-derived leader RNA in both JHM(2)- and JHM(3)-infected cells, whereas all of the subgenomic mRNAs of DECAT2-1 contained the leader RNA derived from the helper virus (Table 2 [compare group IV with group I]; Fig. 4B). These results suggest strongly that the presence of the nine-nucleotide sequence enhanced the transcription efficiency and also allowed the leader RNA to be utilized for the initiation of subgenomic mRNAs.

Similar experiments were performed with 25CAT+9nt, which contained the nine-nucleotide insertion into 25CAT (Fig. 1C). Both 25CAT and 25CAT+9nt yielded the maximum CAT activities (Fig. 3) in both JHM(2)- and JHM(3)-infected cells, consistent with the previous observation that mRNA7 was constitutively expressed (21, 30). However, while the subgenomic mRNA of 25CAT contained a leader RNA derived exclusively from the helper virus (*in trans*), nearly one-third of the mRNAs from 25CAT+9nt contained the DI (*cis*)-derived leader RNA (Table 2) [compare group IV with group I]; Fig. 4A). This result further confirmed the role of the nine-nucleotide sequence in conferring the ability of the leader RNA to initiate mRNA transcription. These data combined indicate that the leaders of both helper virus and DI RNAs regulate and initiate the transcription of subgenomic DI RNA *in trans* and *in cis*.

FIG. 4. Sequences of subgenomic mRNAs derived from constructs with or without the nine-nucleotide sequence. Notation is the same as in Fig. 2. IS7 and IS2-1 indicate the ISs of mRNA7 (A) and mRNA2-1 (B), respectively. (A) mRNA from 25CAT+9nt-transfected and JHM(2)-infected cells. (B) mRNA from DECAT2-1+9nt-transfected and JHM(2)-infected cells.

DISCUSSION

In this study, we have used a differentially expressed IS and various leader sequences in a novel DI-reporter system to demonstrate that the leader RNA of a helper MHV regulates and initiates *in trans* the transcription of DI subgenomic mRNA, thus establishing a critical component of the leader-primed transcription model, i.e., that leader RNA acts *in trans*. Previously, several pieces of data have suggested such a phenomenon. For example, the leader RNA of mRNAs could be switched at a high frequency between two coinfecting viruses (24), and exogenously added leader RNA could be incorporated into mRNAs in an *in vitro* transcription system (2). However, the interpretation of these studies was compromised by the possibility that the viral RNA had undergone recombination; thus, the switched leader RNA could have come from recombinant RNAs. In the present study, some of the plasmids used, such as p25CAT and pDECAT2-1, contain a nine-nucleotide deletion downstream of the leader RNA; this deletion has previously been demonstrated to prevent leader RNA switching or recombination involving DI RNA (22). Furthermore, in a PCR assay, no recombinant RNAs could be detected in this type of DI RNA (16). Thus, the leader sequence of the helper virus and the IS of DI RNA used in this study were unambiguously present in two different RNA molecules. Therefore, the findings that the leader RNA from the helper viral genome was exclusively used for the initiation of CAT-containing mRNAs and also regulated the efficiency of mRNA transcription provide unequivocal evidence that the leader RNA indeed functions *in trans* for subgenomic mRNA

synthesis. This is most consistent with the leader-primed transcription mechanism.

Our studies, surprisingly, identified three different components involved in the regulation of MHV mRNA transcription. The first is the IS, the importance of which has been recognized in numerous analyses of mRNA transcription of coronaviruses (6, 18, 19, 23). Site-specific mutagenesis and deletion studies have further defined the sequence requirement of this region for transcription (6, 18). However, all of the previous studies used a constitutively expressed gene and relied on metabolic labeling of subgenomic mRNAs, which was not sensitive enough for reliable quantitative analysis. In the present study, the use of a differentially expressed IS and a reporter gene allowed us to show that the IS interacts with the leader sequence either directly or indirectly, since the transcription efficiency was influenced by the leader sequences at the 5' ends of both DI RNA and helper viral RNA. It is not clear why the ISs of mRNA7 (UCUAAAC) and mRNA2-1 (UUAUAAAC) behaved differently. Since this stretch of sequence provides the necessary and sufficient transcription initiation signal when it is inserted into DI RNA (6, 18), it functions as a promoter-like sequence for RNA transcription. The second component is the *trans*-acting leader RNA. The sequence of the leader RNA affects in *trans* the transcription efficiency of certain mRNA species, as clearly demonstrated here. Furthermore, the *trans*-acting leader RNA can be incorporated into subgenomic mRNAs. The existence of a *trans*-acting leader RNA during wild-type MHV infection has been postulated by the leader-primed transcription model, which proposes that a free leader RNA is transcribed from the viral RNA, separates from the template and then interacts with the intergenic region to serve as the primer for mRNA synthesis (10). This model best explains the incorporation of the *trans*-acting leader RNA into subgenomic mRNAs. The third component is the *cis*-acting leader RNA, as demonstrated by the finding that the transcription efficiency from the IS of mRNA2-1 was also influenced by the copy number of UCUAA and the presence or absence of the nine-nucleotide sequence immediately downstream of the leader in the DI RNA molecule, even though the leader sequence of DI RNA was not incorporated into subgenomic mRNAs of certain DI constructs. The *cis*-acting leader sequence could exert its function by interacting directly with the IS in the transcription complex; alternatively, the *cis*-acting leader region may be used to transcribe a free leader RNA, which then functions as a *trans*-acting leader. These two mechanisms are not mutually exclusive. These data further support the finding that the *cis*-acting leader sequence functions as an enhancer-like element, independent of its priming function in MHV subgenomic transcription (16).

These findings suggest that these three components, i.e., leader sequence in the DI RNA, leader sequence in the helper viral RNA, and intergenic region, interact with each other during mRNA transcription. These data and those of accompanying report (16) thus suggest a model for MHV mRNA transcription. In this model (Fig. 5), the three RNA components interact with each other via a putative transcription complex formed by cellular proteins and viral RNA polymerase. We propose that the *cis*-acting leader and IS first individually recruit some transcription factors, which in turn bring these RNA elements together via protein-protein interactions. Indeed, we have recently shown that several cellular proteins bind to the 5' end of MHV genomic RNA (3) and IS region (33). The *trans*-acting leader RNA then initiates subgenomic mRNA synthesis at the IS regions. This model best illustrates how a *cis*-acting leader RNA can regulate sub-

FIG. 5. A model for MHV subgenomic mRNA transcription. The small shaded ovals represent viral or cellular factors, and the large shaded oval denotes the putative viral RNA polymerase (Pol.). The thick lines between leader and IS represent the spacer sequence. The postulated transcriptional initiation complex contains a *cis*-acting leader, an IS, a *trans*-acting leader, viral and cellular factors, and viral RNA polymerase. FS indicates leader-mRNA fusion site. The RNA template is of negative sense, present in either single-stranded or double-stranded RNA form.

genomic mRNA transcription even when the *cis*-acting leader is not incorporated into subgenomic mRNAs. It may also explain the previous findings that the transcription of a subgenomic RNA from a DI RNA inhibited DI RNA replication and that this inhibition occurred only in *cis* (5), because the contact between the leader and IS likely prevents RNA replication, which requires uninterrupted RNA synthesis along the whole length of the RNA template. It is uncertain, however, that the interaction among these three RNA components occurs during negative-sense RNA synthesis or mRNA synthesis. Also, the template RNA could be in single-stranded or double-stranded RNA form.

The transcriptional regulation demonstrated in this DI RNA-reporter system matched perfectly with that observed in JHM(2)- and JHM(3)-infected cells (29), suggesting the validity of this system for studying MHV transcription. Nevertheless, the possibility that different subgenomic mRNAs have different translation efficiencies could be raised; thus, the different levels of CAT activity observed for different DI RNAs and helper viruses may be a result of different efficiencies of translation rather than transcription, particularly in view of the fact that mRNAs transcribed from IS7 and IS2-1 contain slightly different lengths of upstream untranslated sequences (Fig. 1B). We consider this possibility unlikely since the CAT activities expressed from IS2-1 and IS7 in JHM(2)-infected cells were similar (Table 1), even though the mRNA structures are slightly different. Also, the mRNAs transcribed from DECAT2-1(3) and DECAT2-1(2) in JHM(3)-infected cells have similar structures and yet have different CAT activities (Table 1). Furthermore, the presence of either two or three UCUAA repeats in the mRNAs did not affect the CAT activity (Tables 1 and 2). In one set of experiments, we have examined

the amount of subgenomic mRNAs by quantitative RT-PCR and confirmed that the CAT activities from these transcripts quantitatively reflected the amount of subgenomic mRNAs (data not shown). These results suggest that differences in CAT activity reflect the differences in transcription efficiency rather than translation efficiency.

The role of the nine-nucleotide sequence (UUUAUAAAC) immediately downstream of the UCUAAAC sequence of the leader is of considerable interest. Previous results showed that the nine-nucleotide sequence did not affect the replication of DI RNA (22). In this study, we have shown that the deletion of the nine-nucleotide sequence significantly reduces the CAT activity (Fig. 3). These results imply that the nine-nucleotide stretch is included in the *cis*-acting sequence required for transcription. Interestingly, none of the leader RNA derived from DI RNAs which have a deletion of these nine nucleotides were incorporated into subgenomic mRNAs, suggesting an alternative possibility that the nine-nucleotide sequence is a transcription terminator for the synthesis of free leader RNAs. Indeed, this region resembles the structure of many eukaryotic transcription terminators (15) in that the 3' end of leader RNA forms a stem-loop structure, followed by the nine-nucleotide sequence, which contains an AU-rich sequence (30). The deletion of this nine-nucleotide sequence may prevent transcription termination of leader RNA; thus, no free leader RNA is synthesized, and the subgenomic mRNA transcription will then exclusively utilize the leader RNA derived from the helper virus. This could also explain why the DI RNA (DIssF) did not undergo leader switching with the helper virus in the absence of the nine nucleotides (22). The insertion of the nine nucleotides may restore the transcription termination, and the free leaders thus generated will participate in subgenomic mRNA transcription and compete with the leaders transcribed from the helper virus RNA (Fig. 5). These data thus are most consistent with the leader-primed transcription mechanism. However, the possibility that discontinuous transcription occurs during negative-strand RNA synthesis (27) still cannot be rigorously ruled out, and the precise nature of the template RNA used for mRNA transcription is yet to be determined. Nevertheless, the studies presented here demonstrated a unique mechanism of a discontinuous transcription which is regulated by RNA sequences both in *trans* and in *cis*.

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