Requirement of the 5'-End Genomic Sequence as an Upstream *cis*-Acting Element for Coronavirus Subgenomic mRNA Transcription

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We have developed a defective interfering (DI) RNA containing a chloramphenicol acetyltransferase reporter gene, placed behind an intergenic sequence, for studying subgenomic mRNA transcription of mouse hepatitis virus (MHV), a prototype coronavirus. Using this system, we have identified the sequence requirement for MHV subgenomic mRNA transcription. We show that this sequence requirement differs from that for RNA replication. In addition to the previously identified requirement for an intergenic (promoter) sequence, additional sequences from the 5' end of genomic RNA are required for subgenomic mRNA transcription. These upstream sequences include the leader RNA and a spacer sequence between the leader and intergenic sequence, which is derived from the 5' untranslated region and part of gene 1. The spacer sequence requirement is specific, since only the sequence derived from the 5' end of RNA genome, but not from other MHV genomic regions or heterologous sequences, could initiate subgenomic transcription from the intergenic sequence. These results strongly suggest that the wild-type viral subgenomic mRNAs (mRNA2 to mRNA7) and probably their counterpart subgenomic negative-sense RNAs cannot be utilized for mRNA amplification. Furthermore, we have demonstrated that a partial leader sequence present at the 5' end of genome, which lacks the leader-mRNA fusion sequence, could still support subgenomic mRNA transcription. In this case, the leader sequences of the subgenomic transcripts were derived exclusively from the wild-type helper virus, indicating that the MHV leader RNA initiates *in trans* subgenomic mRNA transcription. Thus, the leader sequence can enhance subgenomic transcription even when it cannot serve as a primer for mRNA synthesis. These results taken together suggest that the 5'-end leader sequence of MHV not only provides a *trans*-acting primer for mRNA initiation but also serves as a *cis*-acting element required for the transcription of subgenomic mRNAs. The identification of an upstream *cis*-acting element for MHV subgenomic mRNA synthesis defines a novel sequence requirement for regulating mRNA synthesis in RNA viruses.

Several plus-strand RNA viruses, including animal viruses such as Sindbis virus (18, 32) and some plant viruses such as brome mosaic virus (6, 27, 29), cucumber mosaic virus (2), alfalfa mosaic virus (41), and cowpea chlorotic mottle virus (30), synthesize subgenomic mRNAs. In all of these viruses, a single species of subgenomic mRNA is transcribed from a promoter-like sequence present within the genomic RNA in the virus-infected cells. In contrast, mouse hepatitis virus (MHV), a prototype coronavirus containing a single-stranded, positive-sense RNA genome of approximately 31 kb (12, 16), synthesizes six to seven subgenomic mRNAs, each of which initiates from a different site but ends at the common 3' end of the genomic RNA (14, 17). Furthermore, each mRNA contains an identical leader sequence 72 to 77 nucleotides (nt) in length, which is derived from the 5' end of the genome. Several pieces of evidence suggest that each mRNA is transcribed independently; thus, how coronavirus tethers its 5' leader sequence to each mRNA during subgenomic transcription process has been a fascinating and as yet unanswered question. Moreover, the multiplicity of subgenomic mRNAs in coronavirus entails a complex mechanism of transcriptional regulation.

MHV replicates exclusively in the cytoplasm (42). The incoming viral genomic RNA first serves as an mRNA for the expression of a polyprotein, which is processed into multiple

proteins, including RNA polymerase, that carry out transcription and replication (5). The fusion of leader RNA with mRNAs suggests that MHV transcription is likely discontinuous. So far, several discontinuous transcription models have been proposed (12). In the first model, the looping out of the template RNA allows the RNA polymerase complex to jump from the leader to the internal initiation sites (i.e., intergenic sequence [IS] sites) of subgenomic mRNAs. The second model is a posttranscriptional processing mechanism, i.e., a *trans*-splicing mechanism similar to that for trypanosome mRNA synthesis (40). The third model is leader-primed transcription (12), in which a leader RNA is transcribed from one end of the RNA template, dissociates from the template, and then rejoins the template at downstream IS's to serve as a primer for transcription. This model predicts that discontinuous transcription occurs during positive-sense RNA synthesis. The fourth model proposes that discontinuous transcription takes place during negative-sense RNA synthesis (34). This model considers IS elements as transcription terminators for negative-sense RNA synthesis. A considerable body of evidence has suggested that coronavirus could at least, if not only, utilize leader-primed transcription to synthesize its subgenomic mRNAs (reference 12 and references herein) and that the interaction between the leaders and ISs could regulate the expression of at least some viral genes (15, 39, 44, 46).

The inability to manipulate coronavirus genomic RNA has been a major obstacle in studying the molecular mechanism of subgenomic transcription. Recently, the development of repli-

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FIG. 1. Structures of various expression plasmids used. The underlined IS represents the IS derived from the region between genes 6 and 7 of MHV-JHM. The remaining sequence is derived from the vector. The original ATG in the IS is used as the initiation codon for the CAT gene (marked with a bent arrow). The overlined nucleotides are potential termination codons. The long arrow represents the pBluescript vector. The ISCAT fragment was inserted into pDE25 at nt 1491 (marked with an open arrow) in domain II (25), resulting in p25CAT. p25CAT/RIS was identical to p25CAT except for the absence of the IS segment. L denotes the MHV leader, and the solid arrowheads represent the T7 promoter. The thin horizontal lines before and after rectangular boxes denote UTRs.

cating coronavirus defective interfering (DI) RNAs of MHV (25) has made this possible. The prototype of this system has been used to identify the (promoter) sequence requirement for MHV subgenomic transcription (22, 23) and the *cis*-acting sequence for RNA replication (10, 20). We have now developed a refined DI RNA vector which contains a reporter gene, the chloramphenicol acetyltransferase (CAT) gene, placed behind an IS. This system is significantly more sensitive, allowing the quantitative analysis of transcriptional regulation. Using this system, we demonstrate that an upstream *cis*-acting genomic RNA sequence, including a leader RNA, is required for subgenomic mRNA transcription. Moreover, our data also indicate that a partial leader sequence present at the 5' end of genome is sufficient for DI RNAs to initiate subgenomic mRNA transcription, even though the partial leader sequence cannot be incorporated into subgenomic mRNAs. These data strongly suggest that the leader sequence is required for MHV subgenomic transcription both as a *cis* element present at 5' end and as a primer working in *trans*. These findings have significant implications for the mechanism of MHV subgenomic mRNA transcription.

MATERIALS AND METHODS

Viruses and cells. The plaque-cloned A59 strain (26) of MHV and T7 RNA polymerase-vaccinia virus recombinant vTF-3 (7) were used throughout this study. Viruses were propagated in DBT cells (8), a mouse astrocytoma cell line, at a multiplicity of infection of 0.5. DBT cells also were used as recipient cells for RNA transfection experiments.

RNA transcription (RT) and transfection. All plasmid constructs were linearized with *Xba*I, and RNA was transcribed in vitro with T7 RNA polymerase as recommended by the manufacturer (Promega). RNA transfection was carried out by the DOTAP method (Boehringer Mannheim). A59-infected DBT cells were used as the recipient cells for RNA transfection.

Briefly, monolayers of DBT cells at approximately 80% confluence in 6-cm-diameter petri dishes were infected with A59 at a multiplicity of infection of 5. At 1 h postinfection, virus-infected cells were washed once with serum-free Eagle's minimal essential medium (MEM) and then covered with 3 ml of prewarmed MEM containing 7% newborn calf serum. Ten micrograms of in vitro-transcribed RNAs was dissolved in a final volume of 200 μ l of 15% (vol/vol) DOTAP (Boehringer Mannheim) mixture. The RNA-DOTAP mixtures were incubated at 25°C for 15 min and then added slowly to cell cultures. After incubation for 4 h at 37°C, the medium was removed, and cells were washed twice with prewarmed serum-free MEM and further incubated for 5 h (for preparation of cellular extract) or 8 h (for collection of released virus) in MEM containing 2% newborn calf serum. Culture fluid was harvested, clarified of cell debris, and stored at -80°C for further use as the source of virus.

Vaccinia virus infection and DNA transfection. DBT cells in 80% confluency in 6-cm-diameter plates were infected with vTF-3 at a multiplicity of infection of 5. After virus adsorption for 2 h, the infected cells were washed with serum-free MEM, and 3 ml of MEM containing 2% newborn calf serum was added. Ten micrograms of *Xba*I-linearized plasmid DNA was transfected into the infected cells by the DOTAP method as described above, and cells were incubated for 10 h at 37°C.

Plasmid constructions. Plasmid pDE25 (24) was made from DIssE RNA, which is the smallest DI RNA derived from the JHM strain of MHV (25). This construct contains the complete DIssE sequence in genomic sense placed downstream of the T7 polymerase promoter. pDE25 and pCAT-Basic (Promega) were used to construct p25CAT (Fig. 1). pCAT-Basic, containing the CAT gene, was linearized with *Xba*I. The 0.7-kb CAT-containing fragment was generated by PCR using primers 542 (5'-TTACGCCCCGCCCTGCCACTCATCGC-3'), corresponding to the 3' end of the CAT gene open reading frame

FIG. 2. Structures and transcriptional activities of spacer-deletion and spacer-substitution mutants. The broken lines between the boxes depict the deleted regions. The stippled box to the left of each clone represents the MHV leader, and the dark-hatched boxes marked (nt) 1491 denote IS segments. The hatched boxes of LMNCAT and LHDCAT indicate the sequences derived from the nucleocapsid gene of measles virus and from the hepatitis delta virus genome, respectively. Numbers on the right show the relative CAT activities expressed by each clone at different virus passages. P0, CAT activity from cells infected with MHV-A59 and transfected with the given DI RNA; P1, CAT activity from cells infected with the virus derived from P0 cells; P2, CAT activity from cells infected with the virus derived from P1 cells. Values are averages of results from three independent experiments. Dashes indicate that no experiments were done. The diagram was not drawn to scale.

(ORF), and primer 381 (5'-ATTGTTGAGAATCTAATCTAACTTTAAGGATTGGAGAAAAAAT-3'), which contains the IS between genes 6 and 7 (IS7) from MHV-JHM and the first 15 nt of the CAT ORF. This PCR fragment was blunted by T4 DNA polymerase and cloned into the *Sma*I site of pBluescript-SK (Stratagene). The resulting clone is termed pIS-CAT(6/7). pIS-CAT(6/7) was doubly digested with *Spe*I and *Eco*RV, and the CAT-containing fragment, which also contains an extra 15-nt sequence derived from pBluescript-KS, was cloned into the *Spe*I and *Bss*HI sites on pDE25 (Fig. 1), resulting in p25CAT. To construct p25CAT/RIS, the *Spe*I-*Bsp*EI-restricted fragment of p25CAT was replaced by a compatible fragment derived from the *Xba*I-*Bsp*EI-restricted fragment of pCAT-Basic, in which the IS7 region of p25CAT had been eliminated but the CAT gene remained intact. To construct p25ΔStu-Sph, p25CAT was restricted by *Stu*I and *Sph*I, and the large fragment purified from a low-melting-point agarose gel was blunted with T4 DNA polymerase and ligated with T4 DNA ligase. Similarly, p25ΔStu-Spe was made by deletion of the small fragment of *Stu*I-*Spe*I from p25CAT.

To make p541CAT (Fig. 2), p25CAT was first used as a template to PCR generate two fragments, using two pairs of primers: primers 541 (5'-GCAGATCTGTTTAGATTAGATAGATTAGA-3') and -40 (5'-GTTTTCCAGTCACGAC-3'), and primers 538 (5'-GCAGATCTATGGCAAAGATGGCA-3') and 629 (5'-TACTATCAAATCTCTTTAGAC-3').

After digestion with *Bgl*II, both PCR fragments were ligated with T4 DNA ligase, and the ligated fragment was digested with *Cla*I and *Spe*I. Subsequently, the restricted fragment was cloned into the *Cla*I and *Spe*I sites of p25CAT, resulting in p541CAT, which contained a 129-nt deletion between nt 81 and 210 of DIssE. To construct LStuCAT, p541CAT was restricted with *Bgl*II and *Stu*I, and the large fragment isolated from the low-melting-point agarose gel was blunted with T4 DNA polymerase and ligated with T4 DNA ligase. The resulting construct, LStuCAT, had a deletion between nt 81 and nt 480 from DIssE. Similar to the construction of LStuCAT, LSphCAT and LCAT (Fig. 2) were made by deleting from p541CAT segments from nt 81 (*Bgl*II) to 1090 (*Sph*I), and nt 81 (*Bgl*II) to 1491 (*Spe*I) of DIssE, respectively.

To construct LMNCAT and LHDCAT, the DIssE-specific sequence between nt 81 (*Bgl*II) and 1491 (*Spe*I) from p541CAT was replaced by compatible fragments derived from measles virus N gene (1.3 kb of *Bgl*II-*Xba*I fragment) (43) and hepatitis delta virus RNA sequences (0.75 kb of *Bam*HI-*Xba*I fragment) (21), respectively. Similarly, to build LHECAT (Fig. 2), the original spacer of p541CAT was substituted with a compatible fragment of the MHV hemagglutinin-esterase gene (44), which was restricted with *Bam*HI and *Xba*I. To make L6CAT (Fig. 2), a leader-containing fragment of gene 6 (matrix protein) was first reverse transcribed and PCR synthesized from cytoplasmic RNAs of the MHV-JHM virus-infected

FIG. 3. Structures and transcriptional activities of 5'-end-deletion mutants. The diagram notations are the same as in Fig. 2. The relative CAT activities represent the results from P0 cells, and values are averages of results from three independent experiments.

cells, using two primers, 783 (5'-GATTAGATTCTCAACAATACGGTGTCCGCGCCACTC-3') and 540 (5'-GCAAGCTTAATACGACTCACTATAGTATAAGAGTGATTGGCGTCC-3'). The PCR fragment was blunted and cloned into the *EcoRV* site of pBluescript-SK (Stratagene), and a clone with the correct orientation, i.e., containing a *SpeI* site which was located in the 3' end of gene 6, was selected and named pL6. Subsequently, the small restricted fragment of *SnaBI-SpeI* from pL6, containing the entire gene 6, was cloned into the *SnaBI-SpeI* sites of p25CAT, resulting in pL6CAT.

To construct p634CAT (Fig. 3), a PCR fragment from p25CAT was generated by using primers 634 (5'-CGAGATCTATGCCCCGTGGGCC-3') and 629 (see above); the resulting PCR fragment was blunted with T4 DNA polymerase, restricted by *SpeI*, and cloned into the *ClaI* and *SpeI* sites of p25CAT, resulting in p634CAT, which had a 5'-end deletion from nt 1 to 98. Similarly, to make p659CAT (Fig. 3), the PCR fragment amplified from p25CAT by using primers 659 (5'-CGAGATCTGACTTTCGTTCTC-3') and 629 (see above) was cloned into the *ClaI-SpeI* sites of p25CAT. To construct p637CAT (Fig. 3), the PCR fragment amplified from p25CAT by using primers 637 (5'-CGAGATCTACGTGTCCATT-3') and 629 (see above) was cloned into the *ClaI-SpeI* sites of p25CAT. p638CAT was constructed by inserting the PCR fragment amplified from p25CAT by using primers 638 (5'-CGAGATCTGCCCCACCATAGG-3') and 629 (see above) into the *ClaI-SpeI* sites of p25CAT. p538 was constructed by inserting the PCR fragment amplified from p25CAT by using primers 538 (see above) and 629 (see above) into the *ClaI-SpeI* sites of p25CAT.

To build p25CATΔApa I (Fig. 3), p25CAT was restricted by *ApaI*, and the large fragment was religated by T4 DNA ligase. p25CATΔHinc II (Fig. 3) was made by deleting the small fragment from p25CAT restricted by *HincII*.

To generate p25CATΔSnaB I, p25CAT was doubly restricted by *SnaBI* and *ClaI*, and the large restricted fragment was blunted with T4 DNA polymerase and religated by T4 DNA ligase. To construct pL3'ΔCAT, the leader sequence with 3'-end modification was first generated by PCR using two primers, 712R (5'-CTTTGCCATAGATCTGAAACTACAA GAGTTTTAG-3') and -40 (see above). Subsequently, the PCR fragment was doubly restricted by *ClaI* and *BglII*, and the restricted fragment was cloned into the *ClaI-BglII* sites of p541CAT, resulting in pL3'ΔCAT. pL5'3'ΔCAT was made by deleting from the 5' end of pL3'ΔCAT nt 1 to 22, using restriction enzymes *SnaBI-ClaI*, and blunting the restricted ends by T4 DNA polymerase.

CAT assay. Infected and transfected DBT cells in 6-cm-diameter plates were harvested at 8 h posttransfection and lysed by freezing and thawing three times. Cellular lysates were incubated at 60°C for 10 min, and 50 μl of each lysate (from approximately 10⁶ cells) was assayed for CAT activity as previously described (35). Briefly, 50 μl of cell extract was mixed with 65 μl of 0.25 M Tris-HCl (pH 8.0), 4 μl of [¹⁴C]chloramphenicol (0.025 mCi/ml; Dupont NEN), and 5 μl of *n*-butyryl coenzyme A (5 μg/μl; Sigma). After incubation for 8 h at 37°C, the mixture was extracted with 300 μl of xylenes (Mallinckrodt Chemical Works). The phase of xylenes was back-extracted twice with 100 μl of 0.25 M Tris-HCl (pH 8.0), and 200 μl of upper xylenes was measured for ¹⁴C count in a scintillation counter (Beckman Instruments model LS6000IC).

Detection and sequencing of subgenomic CAT mRNAs. To detect CAT mRNAs, cytoplasmic RNAs from A59-infected and RNA-transfected DBT cells were isolated by the Nonidet P-40 method (19) at 8 h postinfection and subjected to RT-PCR, using CAT-specific primer 542 (see above) and leader-specific primer 5'L9 (5'-TGAATGGCGTCCGT-3'). The resulting 0.7-kb PCR fragment was blunted by T4 DNA

TABLE 1. CAT activities of DI vectors upon DNA or RNA transfection

DBT cells	CAT activity ^a
Infected with vTF-3 and transfected with:	
No DNA	1
pISCAT DNA	172
p25CAT DNA	1
p25CAT/RIS DNA	1
pISCAT DNA only (no virus) ^b	1
Infected with MHV-A59 and transfected with:	
No RNA	1
ISCAT RNA	3.3
25CAT RNA	130
25CAT/RIS RNA	1
25CAT RNA only (no virus) ^b	1

^a Expressed as fold increase over the background value. Values are means of the results from three independent experiments.

^b DBT cells were transfected with pISCAT DNA or 25CAT RNA, without prior vTF-3 or MHV-A59 infection.

polymerase and cloned into the *EcoRV* site of pBluescript-SK. The sequences from all constructs were confirmed by chain termination sequencing using dideoxynucleotides (33).

RESULTS

Establishment of a DI vector for studying MHV subgenomic mRNA transcription. To study the transcriptional regulation of MHV mRNAs, we first developed a highly sensitive system for quantitatively detecting subgenomic mRNA synthesis. This system consists of a DI RNA containing the CAT gene placed behind the IS for mRNA7 (termed p25CAT). This plasmid contains two major ORFs; the 5' ORF is derived from the original ORF of DIssE RNA (25), and the 3' ORF is a CAT reporter gene. Between the two ORFs is an IS segment, derived from the IS between genes 6 and 7 (Fig. 1 and Materials and Methods), which has been shown to be a necessary element for mRNA transcription (22, 23). Since the IS segment contains stop codons in every possible reading frame (Fig. 1), the CAT gene cannot be expressed as a DI-fusion protein. The control plasmid, p25CAT/RIS, is identical to p25CAT except that the IS was replaced by an irrelevant sequence derived from the cloning vector. All constructs are under T7 promoter control (Fig. 1).

Since the CAT gene is the second ORF in this DI RNA, it most likely will not be translated (11) unless a separate subgenomic mRNA is transcribed. To establish this point, we carried out the following experiment using a recombinant vaccinia virus expressing T7 RNA polymerase (vTF-3) (7). Cells were infected with vTF-3 and then transfected with various plasmid DNAs, and CAT activity was assayed. In this system, DI RNA was expected to be transcribed by T7 polymerase. Table 1 shows that no CAT activity was detected in cells infected with vTF-3 and transfected with p25CAT or p25CAT/RIS. As a control, pISCAT, in which the CAT gene is the only ORF in the transcript, showed a high CAT activity (172-fold increase). A maximum increase of approximately 500-fold in CAT activity could be reached when more cellular lysates were used. Thus, the CAT activities measured in this study would reflect the amounts of CAT-containing mRNA. This result indicates that the CAT gene product cannot be translated directly from the p25CAT or p25CAT/RIS RNA. In contrast, when the in vitro-transcribed RNAs were transfected into cells infected with a helper MHV, a high CAT activity was detected in 25CAT RNA-transfected cells (130-fold increase;

Table 1). Surprisingly, ISCAT RNA showed only a marginal CAT activity (3.3-fold increase; Table 1), even though the CAT ORF is the only ORF in this RNA. This low CAT activity from ISCAT RNA suggests that RNA transfection efficiency was low; thus, the majority of CAT activity from 25CAT RNA was likely the result of amplification of 25CAT RNA (either replication or transcription or both). Since 25CAT/RIS, which lacks the IS element, did not express CAT activity (Table 1), these results clearly indicate that both MHV helper virus and the IS element are required for CAT expression.

It was previously demonstrated that a subgenomic transcript could be detected from MHV DI RNA which contained an IS element (23). The combined results of RNA transfection and vaccinia virus-induced transcription described above thus strongly suggest that the CAT activity from 25CAT RNA was the result of subgenomic mRNA transcription from the IS site. The presence of a subgenomic CAT-containing mRNA in 25CAT RNA-transfected and MHV-infected cells was confirmed by RT-PCR using a specific set of primers to detect the leader-containing CAT mRNA (see Fig. 5B), thereby establishing that the CAT activity expressed by 25CAT RNA resulted from MHV subgenomic transcription. Thus, the CAT activity expressed by DI RNA constructs can be used as a measure of the efficiency of subgenomic mRNA transcription. This system was then used to explore the sequence requirement for MHV subgenomic transcription.

Dissociation of the sequence requirement for subgenomic transcription from that for RNA replication. The *cis*-acting sequence required for MHV DI RNA replication has recently been determined (10, 20). This sequence includes 470 to 859 nt from the 5' terminus, 135 nt from the internal domain II (Fig. 1) of DI RNA, and 436 nt from the 3' end of the MHV genome. The requirement of this internal sequence may be specific for JHM DI RNA, since two A59 DI RNAs without this internal sequence can replicate (4, 28). We used p25CAT and its derivatives to determine whether the sequence requirement for subgenomic mRNA transcription is the same as that for RNA replication. The in vitro-transcribed 25CAT RNA and other RNAs (Fig. 2) were transfected into MHV-infected cells, and CAT activity was determined in the cellular lysates 8 h after infection (termed P0 cells). First, we found that constructs 25CAT-Stu-Sph and 25CAT-Stu-Spe (Fig. 2), in which the internal replication signal has been deleted (10, 20), expressed relatively high levels of CAT activity, although they were lower than that of the wild-type 25CAT RNA (Fig. 2). Since these two mutant RNAs did not contain part of the 5' end and the internal sequence required for JHM DI RNA replication (10, 20), they were not expected to replicate. To confirm this point, the supernatant virus from the transfected cells (P0 cells) were used to infect fresh DBT cells (P1 cells); no CAT activity was detected in P1 or in the subsequent passage (P2). In contrast, 25CAT, which contained all of the RNA replication signals, showed high levels of CAT activity in both P1 and P2 cells (Fig. 2). In addition, both the genomic RNA and the subgenomic CAT mRNA could be detected by Northern (RNA) blotting in the P1 cells infected with 25CAT-containing virus (data not shown), indicating that 25CAT RNA could replicate and be passaged despite the absence of a specific packaging signal. In contrast, DI and subgenomic mRNAs of 25CAT-Stu-Sph and 25CAT-Stu-Spe could be detected in P0 cells but not in P1 cells, suggesting the absence of RNA replication (data not shown). These results indicate that subgenomic transcription can occur in the absence of RNA replication. Thus, the CAT activity detected in P0 cells of these two RNAs was the result of the transcription of a CAT-containing subgenomic mRNA. Therefore, the previously

characterized internal replication signal and a substantial portion of the 5'-end replication signal (10, 20) are not required for subgenomic mRNA transcription. We next examined whether the 5'-end 480 nt are essential for MHV subgenomic transcription. We constructed LStuCAT (Fig. 2), which had a deletion of the 400 nt downstream of the leader sequence. This deleted region has been shown to be required for the replication of all natural and synthetic MHV DI RNAs characterized so far (4, 10, 20, 28). Interestingly, this RNA also expressed a high CAT activity in P0 cells but no activity in P1 or P2 cells, indicating that this 400-nt deletion abolished replication ability but did not affect transcription capability. Taken together, these experiments clearly reveal that the sequence requirement for MHV DI subgenomic transcription differs from that for RNA replication. The finding that the replication-competent 25CAT RNA did not yield a significantly higher CAT activity than the replication-incompetent mutant RNAs is not unexpected: it has been demonstrated that the presence of internal IS segment in the DI RNA significantly reduces DI RNA replication (9).

The requirement of the spacer sequence between the leader and IS for subgenomic transcription. The results presented above showed that neither half of the spacer sequence between the leader and IS segment is required for mRNA transcription. We next deleted the entire sequence from nt 81 to 1491 (LCAT; Fig. 2). Surprisingly, practically no CAT activity was detected, indicating that some spacer sequence is important for subgenomic transcription. To determine which region of the spacer is required for MHV subgenomic transcription, we made serial deletions of different sizes in the spacer sequence (Fig. 2). Clone 541CAT (Fig. 2), with a deletion of nt 81 to 210, expressed almost the same level of CAT activity as wild-type 25CAT. Similarly, LStuCAT (Fig. 2), which has a deletion from nt 81 to 480, also expressed a high CAT activity. However, a further deletion of the spacer sequence from nt 81 to 1090 (LSphCAT) drastically reduced CAT activity to only a 7.5-fold increase. These results suggest that the sequence between nt 480 and 1090 is important for mRNA transcription. However, as previously noted, p25CAT Δ Stu-Sph and p25CAT Δ Stu-Spe (Fig. 2), with deletions of nt 480 to 1090 and nt 1491, respectively, retained most of their CAT activities. Thus, the spacer sequence requirement for mRNA transcription is either redundant or nonspecific. It should be noted that 541CAT retained some CAT activity in P1 but not P2 cells. The residual CAT activity in P1 cells was probably due to the high level of mRNA expression of 541CAT in P0 cells, which was passaged nonspecifically by virus particles.

It should be pointed out that the structures of the CAT-containing subgenomic mRNAs of all of these constructs are identical, regardless of the structure of the upstream sequence. Therefore, the differences in their CAT activities could not be due to possible differences in translation efficiency of mRNA. Also, the differences in CAT activity could not be due to differences in the length of the DI-specific ORF at the 5' ends of these constructs, inasmuch as there was little correlation between the length of this upstream ORF and CAT activity.

To determine whether the spacer sequence requirement is specific, we substituted the spacer sequence, which is derived from MHV gene 1 and 5'-end untranslated region (UTR), with various other sequences. LMNCAT and LHDCAT (Fig. 2) contained a part of measles virus N gene (1.3 kb of *Bgl*II-*Xba*I fragment) (43) and of hepatitis delta virus sequences (0.75 kb of *Bam*HI-*Xba*I fragment) (21), respectively. The results showed that neither of these RNAs expressed any CAT activity. We also substituted the DI spacer sequence with the coding sequence of MHV-JHM gene 6 (M protein) in L6CAT

(31) and the hemagglutinin-esterase gene in LHECAT (44). Neither of these two clones expressed any CAT activity (Fig. 2). These results strongly suggest that the requirement of a spacer sequence between the leader and IS for subgenomic mRNA transcription is specific and that only the sequence derived from MHV gene 1 and 5' UTR can serve as a functional spacer. This required sequence is probably redundant within the 5' UTR and gene 1. It is significant to note that the structure of L6CAT is almost identical to that of MHV mRNA6. The finding that it did not express any CAT activity strongly suggests that the wild-type MHV subgenomic mRNAs and/or their negative-strand counterpart RNAs cannot be used for RNA transcription.

MHV subgenomic transcription requires the 5'-end leader sequence as a *cis* element. We next determined whether the leader sequence was also required as a *cis* element for MHV subgenomic transcription. To address this issue, a panel of 5'-end-deletion mutants was constructed as shown in Fig. 3. Interestingly, the total deletion of the leader sequence drastically reduced the CAT activity; however, most of the mutants with a deletion from the 5' end up to nt 210 still demonstrated basal levels of CAT activity in P0 cells, ranging from 3.9-fold for 538CAT to 7.5-fold for 638CAT. However, when the 5'-end deletions extended beyond position 210, which is the translation start site for MHV gene 1, the resulting clones of 25CAT Δ Apa I and 25CAT Δ Hinc II (Fig. 3) completely lost CAT activity. These data suggest that the leader sequence is not required for basal-level subgenomic transcription. However, the comparison between 25CAT and 634CAT RNAs, LStuCAT and 25CAT Δ Hic II RNAs, and 541CAT and 538CAT RNAs showed that the inclusion of a leader sequence clearly augmented the CAT activity to almost the wild-type 25CAT level. As a control, 25CAT/RIS, which contained the leader but no IS element, did not express any CAT activity (Fig. 3), indicating that both the leader and IS are required in the same RNA molecule for subgenomic mRNA transcription. The stabilities of the transfected RNAs, including the leaderless RNAs and wild-type 25CAT RNA, present in the infected cells were approximately the same, as judged by Northern blot analysis using a CAT-specific probe (data not shown). The results of these experiments clearly demonstrated that the leader sequence of 25CAT is required as a *cis* element for efficient MHV subgenomic transcription. However, leader RNA alone is not sufficient for subgenomic mRNA transcription, as shown by the lack of CAT activity in some leader-containing DI RNAs such as LCAT and LSphCAT (see above). This result also suggests that nt 210 to 248 are required for basal transcription activity.

To further determine whether the entire leader sequence is needed for MHV subgenomic transcription, we made three partial-deletion mutants of the leader: L3' Δ CAT, 25CAT Δ SnaB I, and L5'3' Δ CAT (Fig. 4). L3' Δ CAT had a deletion of 20 nt at the 3' end of the leader, which contains a palindrome sequence of four pentanucleotide repeats (AAAUCUAA UC UAA UCUAA UCUAAA). This sequence is thought to interact with the downstream IS elements to generate leader-mRNA fusion (12). Interestingly, L3' Δ CAT RNA still expressed CAT activity at almost the wild-type level (Fig. 4). Another clone, 25CAT Δ SnaB I, had a deletion of the 5'-most 22 nt of the leader sequence and yet expressed a wild-type level of CAT activity (Fig. 4). Moreover, L5'3' Δ CAT RNA, which contains only nt 22 to 60 of the leader, expressed a significant level of CAT activity (11-fold increase over that of 538CAT RNA) (Fig. 4). These results indicate that even a partial leader sequence at the 5' end of the genome allowed subgenomic mRNA transcription, even though this partial leader had lost

FIG. 4. Structures and transcriptional activities of leader-modified mutants. The relative CAT activities represent the results from P0 cells, and values are averages of the results from three independent experiments. Other diagram notations are the same as in Fig. 2.

the ability to serve as a primer to be physically joined to subgenomic mRNAs (see below). These results taken together suggest that the MHV leader in *cis* is required for promoting subgenomic transcription.

To rule out the possibility that recombination between DI RNAs and helper virus RNA had restored the leader sequence in these DI RNAs, we performed the following RT-PCR experiment to detect any possible recombinant RNAs present in the cells transfected with these DI RNAs (Fig. 5). The 3' antisense primer (630; Fig. 5A) used is specific for DI RNAs and would not detect helper virus A59 sequence (see the legend to Fig. 5). The 5' primer (5'L9; Fig. 5A) corresponds to the leader sequence of both DI and helper virus RNAs. The 25CAT RNA and any recombinant RNA with a restored leader sequence should yield a PCR product 880 nt in length. Figure 5B shows that only 25CAT RNA generated the 880-nt PCR product. Constructs L3'ΔCAT and LStuCAT RNAs (Fig. 5B, lanes 3 and 4), both of which contain an intact 5' end of the leader, generated 480- and 860-nt PCR products, respectively, which correspond to the sizes of the original DI RNA constructs. No PCR products of larger size were detected in these two and any other RNAs, which did not have intact 5' ends of the leader sequence. Therefore, we conclude that recombination between DI RNAs and helper virus RNA did not occur in these cases.

The leader sequences of CAT-containing subgenomic mRNAs are derived in *trans*. Since the subgenomic mRNAs derived their leader sequence from the 5' end of genomic RNA (13), it was of interest to determine the structures and origins of the leader sequences in subgenomic mRNAs transcribed from DI RNAs with partial leader sequences. For this purpose, we performed RT-PCR using two specific primers to amplify the leader-containing CAT mRNAs from P0 cells, which had been transfected with the various RNAs. Three constructs, 25CATΔSnaB I, L3'ΔCAT, and L5'3'ΔCAT, all of which have a partially deleted leader, were analyzed, with

FIG. 5. Screening of possible recombinants between DI and helper virus RNA. (A) Schematic diagram of primers used in RT-PCR. The 3' primer 630 (5'-ATATACAACATCTGTTGCATT-3') is specific for the junction between domains I and II in 25CAT (Fig. 1). The 5' primer 5'L9 (corresponding to nt 9 to 22 from the 5' end) is a leader-specific primer. PCR conditions were 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min, for 30 cycles. The size of the predicted product from the possible recombinants is 880 nt. (B) Agarose gel electrophoresis of PCR products stained with ethidium bromide. Lane M: ϕ X174 DNA-*Hae*III markers. Cytoplasmic RNAs used as templates for RT-PCR were isolated from A59-infected DBT cells transfected with 25CAT (lane 1), L3'ΔCAT (lane 2), LStuCAT (lane 3), 25CATΔSnaB I (lane 4), L5'3'ΔCAT (lane 5), 634CAT (lane 6), 538CAT (lane 7), and 25CATΔApa I (lane 8). The 880-nt PCR product is marked with an arrow.

25CAT as a control. The 3' primer used in the RT reaction was specific for the CAT gene at its 3' end, and the 5' primer was specific for the consensus sequence (nt 9 to 22, which is present in all of the DI RNA constructs) of the leader (Fig. 6A). The predicted size of the PCR product originating from the possible leader-containing CAT mRNA was 760 nt. The results showed that all three constructs and the control 25CAT generated a PCR band of the correct size predicted for CAT mRNAs (Fig. 6B), indicating that all of them indeed initiated subgenomic CAT mRNA transcription from the IS sites. The predicted PCR products from the full-length DI RNAs (ranging from 1,900 to 2,100 nt) were not detected, probably because of poor PCR efficiency for larger RNAs. The PCR products were cloned, and independent cDNA clones were sequenced. Since there is a difference of five nucleotides in the leader sequences between the helper virus A59 and 25CAT RNA (Fig. 6C, sequence a), the origin of the leader in the subgenomic mRNAs could be determined with certainty. The results demonstrated that the leader of CAT mRNAs from all clones, including six from 25CAT, six from L3' Δ CAT, five from 25CAT Δ SnaB I, and six from L5'3' Δ CAT, were derived from the helper virus A59 (Fig. 6C, sequence b). Since the 5' primer (5'L9; Fig. 6A) used in this RT-PCR did not discriminate the leaders from DI or helper virus RNA, the results clearly indicate that the defective leaders of DI RNA cannot serve as a primer to initiate transcription. This result thus firmly establishes that the leader RNA of subgenomic mRNA is derived in *trans*.

The sequence alignment between the leader and IS (Fig. 6C) demonstrated that the fusion sites between the leader and IS of CAT mRNAs from all of the RNAs were similar. In almost all of them, fusion site between leader and transcript is located within the consensus IS sequence, resulting in either two or three repeats of UCUAA pentanucleotides. However, in one clone, the leader-mRNA fusion site is located upstream of the consensus repeats. This fusion mechanism for this mRNA species is not clear. This type of aberrantly fused mRNAs has recently been found to occur frequently in some MHV strains (unpublished observation). Since the leader sequences of these mRNAs were derived exclusively from the helper viral RNA, we conclude that while the defective leader RNA cannot serve as a physical primer for mRNA transcription, it nevertheless can enhance transcriptional activities in *cis*. Taken together, these results establish that the leader RNA provides *cis*-acting functions, which are separate from its postulated priming function, for MHV subgenomic transcription.

DISCUSSION

Our current studies demonstrate that MHV subgenomic mRNA transcription requires not only an IS segment, which is equivalent to a promoter sequence (transcription start site), but also an upstream leader and 5'-end genomic sequence on the same RNA molecule. In the absence of the *cis*-acting leader sequence, only a basal level of subgenomic mRNA transcription occurs, even though the leader RNA sequence and all of the necessary sequences are present in the coinfecting viral RNA. Furthermore, the *cis*-acting leader sequence is not necessary for the priming function, since it is the helper viral leader RNA which is physically joined to subgenomic mRNAs. Thus, the *cis*-acting leader and 5'-end genomic sequence most likely perform regulatory functions in subgenomic mRNA synthesis; in contrast, IS is the promoter-like sequence, since it is absolutely required for RNA transcription and is the transcription initiation site. How the *cis*-acting upstream sequence regulates MHV subgenomic mRNA syn-

FIG. 6. Detection of subgenomic CAT mRNAs. (A) Schematic diagram of primers used in RT-PCR for the detection of subgenomic CAT mRNAs. The size of the predicted product from the subgenomic transcript is 0.7 kb, and that from the full-length DI RNAs ranges from 1.9 to 2.1 kb. (B) Agarose gel electrophoresis of PCR products stained with ethidium bromide. Lane M, ϕ X174 DNA-HaeIII markers. Cytoplasmic RNAs used as templates for RT-PCR were isolated from A59-infected DBT cells that were mock transfected (lane 1) or transfected with 25CAT (lane 2), L3' Δ CAT (lane 3), L5'3' Δ CAT (lane 4), or 25CAT Δ SnaB I (lane 5). The PCR products of 0.7 kb are marked with an arrow. (C) Sequence analysis of the leader regions from the 0.7-kb bands cloned from panel B, which represent the CAT mRNAs. a, leader sequence alignment between MHV-A59 and 25CAT. The nucleotide differences are marked with nucleotide numbers from the 5' end of the genome. b, sequence alignment between leaders of subgenomic CAT mRNAs and IS7. The sequence on the top of each comparison represents IS7; bent arrows indicate the start codons of CAT gene; the underlined sequences identify the UCUAA pentanucleotide repeats; the sequences with asterisks represent the consensus IS core sequence.

thesis is not clear. It is possible that the leader sequence in *cis* affects the synthesis of negative-sense RNA templates used for MHV subgenomic transcription. This is, however, unlikely since we have recently shown that all of these RNAs synthesize

comparable amounts of negative-strand RNA (20a). Alternatively, the leader sequence in *cis* functions in a manner similar to DNA-dependent RNA transcriptional enhancers. Indeed, several properties of the *cis*-acting leader sequence support the notion that it may function as an enhancer. First, even in the absence of the leader sequence, there was still a basal level of transcription; however, the presence of the *cis*-acting leader sequence significantly enhanced the transcriptional activity. The presence of the *cis*-acting leader sequence in the absence of the IS did not activate transcription at all. Second, the *cis*-acting leader regulates transcription initiation from IS elements from a very long, variable distance. In the present study, the distances between the leader and IS segments range from approximately 0.4 to 1.4 kb in the DI RNAs capable of transcribing subgenomic mRNAs. In the MHV genome, the leader is 22 kb away from the nearest promoter (IS) (16). Thus, the characteristics of long-range action and *cis*-acting behaviors make the MHV leader reminiscent of the DNA enhancer for RNA transcription.

The upstream *cis*-acting RNA elements identified here are also reminiscent of the TAR RNA of human immunodeficiency virus and the RNA stem-loop from the *nut* site of the λ phage, since all of these involve RNA-mediated regulation of transcription (for reviews, see references 3 and 38); i.e., they are *cis*-acting RNA molecules that, in conjunction with viral and cellular factors, activate transcription. However, the MHV leader sequence is present upstream, rather than downstream, of each promoter (IS) element. Furthermore, the first TAR and *Nut* RNA have to be transcribed from DNA promoter, and it is the RNA transcript which enhances subsequent transcription from the same promoter. Thus, MHV leader RNA is more similar to DNA enhancers in that it is part of the template and directly regulates transcription initiation from the promoter (IS) sites. These unique characteristics make MHV leader RNA a new type of *cis*-acting RNA element that mediates transcription activation.

It should be noted that the leader sequence alone plus IS is not sufficient for initiating mRNA transcription; some spacer sequence from the 5' UTR and MHV gene 1 is also required. Thus, the interaction between the leader and IS element is probably mediated through another stretch of sequence. The spacer sequence required is specific for the 5' UTR and gene 1 sequences. However, two different regions of the gene 1 (i.e., nt 210 to 480 and nt 480 to 1491 from the 5' end of the genome) could substitute for each other in providing the necessary spacer sequence. This finding suggests that the necessary sequences are redundant.

The requirement of the upstream regulatory sequences for MHV subgenomic mRNA transcription is novel among RNA viruses. In the case of brome mosaic virus, an earlier study (29) demonstrated that the sequence between 20 nt upstream (-20) and 16 nt downstream (+16) of the subgenomic RNA initiation site constituted a promoter sequence (core promoter) for subgenomic mRNA synthesis. Recent studies on the requirement of upstream sequences, however, showed that this 37-nt core promoter provided only a basal level of subgenomic transcription and that an additional sequence of approximately 55 nt located immediately upstream of the core promoter was essential for a normal level of subgenomic transcription (6, 27). In contrast to brome mosaic virus, the *cis*-acting upstream sequences for MHV subgenomic mRNA transcription identified here are far away from the nearest IS site (promoter) on the genomic RNA. Conceivably, coronavirus may have evolved such a unique genetic arrangement between its upstream sequences and IS sites (promoters) so

that its multiple ORFs can be properly expressed during viral replication.

The requirement of gene 1 and 5' UTR sequences as spacer sequences for subgenomic mRNA transcription strongly suggests that the wild-type viral subgenomic mRNAs (mRNA 2 to mRNA 7) and their counterpart subgenomic negative-strand RNAs cannot be used as templates for mRNA transcription, since the 5' UTR and gene 1 sequences are present in the genomic RNA but not in subgenomic mRNAs. It has been proposed that subgenomic positive- and negative-sense RNAs serve as templates for further amplification of subgenomic mRNAs through either a transcription or replication mechanism (36, 37). However, it has been shown that MHV RNA replication requires the 5'-end 470 to 859 nt, which are missing from the subgenomic mRNAs (10, 20). Thus, these subgenomic mRNAs are unlikely to serve as replicons. In this study, we have further shown that the LCAT RNA (Fig. 2), which is very similar to MHV mRNA7 in structure, failed to transcribe mRNAs. Furthermore, L6CAT RNA (Fig. 2), which resembles MHV mRNA6, also could not be transcribed. In addition, when the spacer sequence of 25CAT RNA was replaced by heterologous sequences, such as the measles virus N gene, hepatitis delta virus RNA, or MHV hemagglutinin-esterase gene sequence (Fig. 2), subgenomic mRNA transcription also could not occur. These findings strongly indicate that only the genome-size viral RNA, which contains the 5' UTR and entire gene 1 sequences, can be transcribed into mRNAs and that subgenomic mRNAs can neither replicate nor transcribe. Considering all of these findings, we conclude that MHV uses only the genome-size viral RNA to initiate subgenomic mRNA transcription during wild-type viral infections. This conclusion is consistent with the result obtained by Yokomori et al. (45), which showed that a genome-length RNA template is required for MHV subgenomic mRNA synthesis at least early in infection. However, whether the genome-size negative-strand template is used for transcription throughout the viral replication cycle remains to be determined.

The essence of the leader-primed transcription model is that the free leader RNAs found in the MHV-infected cell (1) could initiate subgenomic mRNA transcription in *trans*. Indeed, our data strongly support this notion, because the leader sequences in the CAT mRNAs from all of the DI RNAs studied (including 25CAT RNA; Fig. 4 and 5) were derived from the helper virus. Conceivably, the leaders in these DI RNAs are defective, and thus cannot be used as primers, but retain the enhancer function for subgenomic transcription. Thus, these data clearly demonstrate that the *trans*-acting priming function of the leader RNA and its function as a *cis*-acting element for mRNA transcription are separable. To be a primer, the leader sequence must preserve the 3'-end sequence to interact with the downstream IS elements. In contrast, to be a *cis*-acting element for subgenomic transcription, the leader sequence requirement is somewhat more flexible. Therefore, the roles of the MHV leader in wild-type viral infection are twofold: to provide genetic information for the synthesis of free *trans*-acting leaders and to serve as a *cis*-acting element for subgenomic mRNA transcription.

As demonstrated by its ability to express a foreign gene, the DI RNA system described here may be useful as a vector for the expression of foreign genes when coinfecting with a helper virus. This ability will be useful for studying MHV pseudotyping or the effects of certain foreign genes on MHV infection in cell cultures or in animals. Since this system utilizes a homologous DI RNA that can be activated only by MHV, the foreign gene will be expressed only in the virus-infected cells, thus

allowing the studies of the specific effects of foreign genes on viral infection.

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