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Evidence for Coronavirus Discontinuous Transcription

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Coronavirus subgenomic mRNA possesses a 5′-end leader sequence which is derived from the 5′ end of genomic RNA and is linked to the mRNA body sequence. This study examined whether coronavirus transcription involves a discontinuous transcription step; the possibility that a leader sequence from mouse hepatitis virus (MHV) genomic RNA could be used for MHV subgenomic defective interfering (DI) RNA transcription was examined. This was tested by using helper viruses and DI RNAs that were easily distinguishable. MHV JHM variant JHM(2), which synthesizes a subgenomic mRNA encoding the HE gene, and variant JHM(3-9), which does not synthesize this mRNA, were used. An MHV DI RNA, DI(J3-9), was constructed to contain a JHM(3-9)-derived leader sequence and an inserted intergenic region derived from the region preceding the MHV JHM HE gene. DI(J3-9) replicated efficiently in JHM(2)- or JHM(3-9)-infected cells, whereas synthesis of subgenomic DI RNAs was observed only in JHM(2)-infected cells. Sequence analyses demonstrated that the 5′ regions of both helper virus genomic RNAs and genomic DI RNAs maintained their original sequences in DI RNA-replicating cells, indicating that the genomic leader sequences derived from JHM(2) functioned for subgenomic DI RNA transcription. Replication and transcription of DI(J3-9) were observed in cells infected with an MHV A59 strain whose leader sequence was similar to that of JHM(2), except for one nucleotide substitution within the leader sequence. The 5′ region of the helper virus genomic RNA and that of the DI RNA were the same as their original structures in virus-infected cells, and the leader sequence of DI(J3-9) subgenomic DI RNA contained the MHV A59-derived leader sequence. The leader sequence of subgenomic DI RNA was derived from that of helper virus; therefore, the genomic leader sequence had a *trans*-acting property indicative of a discontinuous step in coronavirus transcription.

Mouse hepatitis virus (MHV), a coronavirus, is an enveloped virus with a single-stranded, positive-sense RNA genome of approximately 31 kb (14, 15, 27). Seven to eight species of virus-specific mRNAs, which make up a 3′-coterminal nested set, are made in MHV-infected cells (12, 16). They are named, in decreasing order of size, mRNAs 1 through 7 (12, 16). The 5′ end of the MHV genomic RNA and the mRNAs starts with a 72- to 77-nucleotide-long leader sequence (11, 13, 34). Interestingly, the leader sequence is encoded only once in the genomic RNA, at the 5′ end. On the subgenomic mRNAs, the leader sequences are fused with the mRNA body sequence. The mRNA body sequences begin from a consensus region of the intergenic sequence, which is located upstream of each MHV gene (7, 11, 13, 22, 34).

MHV subgenomic RNAs are not detected in MHV virions; therefore, at some point in the virus's replication cycle, they must be synthesized from a full-length genome (14, 35) and the leader RNA must become fused at the 5′ end of each mRNA. Subgenomic negative-strand RNAs, each of which corresponds to subgenomic mRNA, are present in coronavirus-infected cells (32). One coronavirus, transmissible gastroenteritis virus, has an antileader sequence at the 3′ end and a short poly(U) sequence at the 5′ end of its subgenomic negative-strand RNA (31, 32). There are at least two stages in coronavirus subgenomic RNA synthesis: one is primary transcription, during which subgenomic-size RNA is synthesized from a genomic-size template RNA; the other is secondary transcription, for which subgenomic-size RNA serves as the template (6). All of the activities necessary for MHV RNA synthesis are present continuously during the first 6 h of infection (6).

Several models have been proposed to explain coronavirus primary transcription. One is leader RNA-primed transcription. This proposes that a leader RNA is transcribed from the 3′ end of the genomic-size, negative-strand template RNA, dissociates from the template, and then rejoins the template RNA at downstream intergenic regions to serve as the primer for mRNA transcription (1, 3, 4, 9). Another model states that subgenomic negative-strand RNAs are initially synthesized from the input genomic RNA (30). The antileader sequence may be acquired during subgenomic negative-strand RNA synthesis by looping out of the positive-strand genomic RNA or a mechanism similar to *cis* splicing. Three other suggested mechanisms of subgenomic mRNA synthesis involve looping out of the genomic-size negative-strand RNA and *cis*- or *trans*-splicing-like mechanisms (4).

The mechanism of coronavirus secondary transcription is also obscure. It was proposed that coronavirus secondary transcription may be involved in the replication of each subgenomic RNA species (32). Although not consistent with some published data (8, 16–18), this mRNA-replicon model is consistent with the presence of subgenomic replicative intermediate RNAs corresponding to each MHV subgenomic mRNA species in MHV-infected cells (30).

Evidence supportive of leader RNA-primed transcription is that during a mixed infection with two MHVs, the leader RNA sequences from one virus appear, at high frequency, on mRNAs 6 and 7 of the coinfecting virus (23). These leader-reassorted mRNA species in coinfected cells suggest that leader RNA species possess a *trans*-acting property. This interesting conclusion needs to be carefully reexamined, however, because coronavirus undergoes high-frequency homologous RNA-RNA recombination (2, 19). During mixed infection, large numbers of recombinant MHV genomes may be

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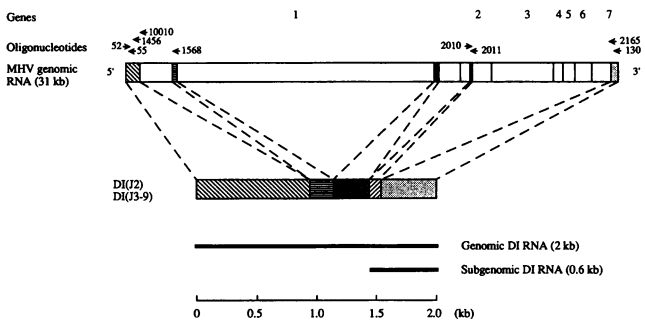

FIG. 1. Schematic diagram of the structure of DI(J2) and DI(J3-9). The structure of DI(J2) and DI(J3-9) is compared with that of the standard MHV genomic RNA. The structures of the genomic and subgenomic DI RNAs of DI(J2) and DI(J3-9) are also shown. Genes 1 through 7 represent the seven genes of MHV. The names of all of the oligonucleotides used in this study are shown above the diagram of MHV genomic RNA, and the locations of their genomic RNA-binding sites are indicated with arrows.

generated. If these contain odd numbers of crossovers and the leader RNA works only in *cis*, then many subgenomic mRNA molecules would consist of the reassorted structure; the leader and the body sequence of mRNA would not be derived from different viruses.

In this study, we reexamined whether the MHV leader sequence acts in *trans* during MHV RNA transcription. We used the MHV defective interfering (DI) RNA system from which a subgenomic DI RNA, initiated at an inserted intergenic region, is synthesized. After transcription, we clearly saw the leader sequence from the helper virus on the 5′ end of a subgenomic DI RNA. This reassortment of MHV leader RNAs was distinct from recombination and indicates a *trans*-acting function of MHV leader RNA in transcription.

MATERIALS AND METHODS

Viruses and cells. The plaque-cloned A59 strain of MHV (12) and two plaque-cloned MHV JHM variants, JHM(2) and JHM(3-9), were used as helper viruses. JHM(2) contains two UCUAA repeats with a nine-nucleotide sequence stretch, UUUAUAAAC, at the junction between the leader and the remaining body sequence (20, 33). JHM(3-9), which contains three UCUAA repeats without the nine-nucleotide stretch, was isolated after 29 consecutive undiluted passages of the original plaque-cloned JHM strain. Mouse DBT cells (5) were used for both RNA transfection and propagation of viruses.

Plasmid construction. The names of all oligonucleotides and the locations of their MHV genome-binding sites are shown in Fig. 1. MHV JHM-specific cDNA was synthesized from intracellular RNA of JHM(2)-infected cells as previously described (20, 22). Oligonucleotide 2011 (5′-AGCCATAGATATCC ACGTACT-3′), which has an *Eco*RV site and hybridizes at nucleotides 868 to 888 from the 5′ end of gene 2, was the primer (33). This MHV-specific cDNA was used in a PCR with oligonucleotide 2010 (5′-ATCTATTTTGGTACCGAT TG-3′), which contains a *Kpn*I site and binds to negative-sense MHV RNA at nucleotides 773 and 793 of gene 2. The reaction took place in PCR buffer (0.05 M KCl, 0.01 M Tris-HCl [pH 8.3], 0.0025 M $MgCl_2$, 0.01% gelatin, 0.17 mM each deoxyribonucleoside triphosphate, 5 U of *Taq* polymerase [Promega]) at 93°C for 45 s, 37°C for 30 s, and 72°C for 100 s for 25 cycles. The reverse transcriptase (RT)-PCR product was digested with *Kpn*I and *Eco*RV and inserted into the 3.6-kb-long *Kpn*I-*Eco*RV fragment of PR6 (18), yielding PF2b.

Two virus-specific cDNAs were synthesized from intracellular RNA of JHM(2)- and JHM(3-9)-infected cells by using as the primer oligonucleotide 1456 (5′-ACTACCGAACTGC AATGC-3′), which hybridizes at nucleotides 490 to 508 from the 5′ end of the genome (15). RT-PCR products resulted from incubation of each cDNA product with oligonucleotide 52 (21), which hybridizes to negative-strand MHV genomic RNA at nucleotides 1 to 24 from the 3′ end under the PCR conditions described above. The 0.5-kb *Sna*BI-*Stu*I fragment of the JHM(2) RT-PCR product and that of the JHM(3-9) RT-PCR product were inserted into the 3.2-kb *Sna*BI-*Stu*I fragment of PF2b, yielding PF2RWT and PF3RWT, respectively.

Deletion of a 1.7-kb-long *Sph*I-*Acc*I fragment located between nucleotides 1085 and 2792 of clones PF2RWT and PF3RWT yielded plasmids DI(J2) and DI(J3-9), respectively. The regions derived from insertion of the RT-PCR product were completely sequenced to confirm the presence of specific mutations and the absence of extraneous mutations. The structures of DI(J2) and DI(J3-9) are shown in Fig. 1.

RNA transcription and transfection. Plasmid DNAs were linearized by *Xba*I digestion and transcribed with T7 RNA polymerase (19). The transcribed RNA was transfected by lipofection (19).

Radiolabeling of viral RNAs and agarose gel electrophoresis. Virus-specific RNAs in virus-infected cells were labeled with $^{32}P_i$ (24) and separated by electrophoresis on 1% agarose gels after denaturation with 1 M glyoxal (26).

Preparation of virus-specific intracellular RNA and Northern (RNA) blotting. Virus-specific RNAs were extracted from virus-infected cells (24). For RT-PCR analysis of negative-strand RNAs, total RNA was extracted as described previously (8). For each sample, 1.5 μg of intracellular RNA was denatured and electrophoresed through a 1% agarose gel containing formaldehyde, and the separated RNA was blotted onto nylon filters (18). The RNA on the filters was hybridized with ^{32}P-labeled probes specific for the 3′ and 5′ ends of MHV (28). The probes were prepared from the gel-purified 0.25-kb *Nru*I-*Msc*I fragment of DF1-2 (25), which corresponds to a region 18 to 262 nucleotides from the 3′ end of MHV DI cDNA, and from the 0.61-kb *Stu*I-*Sph*I fragment of DF1-2, which corresponds to a region 481 to 1091 nucleotides from the 5′ end of MHV DI cDNA.

RT-PCR. For amplification of positive-strand subgenomic DI RNA species, MHV-specific cDNA was synthesized from intracellular RNA by using as a primer oligonucleotide 130 (5′-TTCCAATTGGCCATGATCAA-3′), which binds to positive-strand MHV RNA at nucleotides 8 to 27 from the 3′ end. After cDNA synthesis, MHV-specific cDNA was incubated with oligonucleotide 52 in PCR buffer at 93°C for 45 s, 37°C for 30 s, and 72°C for 100 s for 25 cycles. For amplification of negative-strand subgenomic DI RNA species, cDNA was synthesized by using oligonucleotide 52. This cDNA was incubated with oligonucleotide 130 by using the same PCR conditions. For amplification of genomic DI RNA, oligonucleotide 1568 (5′-TCTGCATATGCAACATC-3′), which binds to positive-strand DI RNA at nucleotides 871 to 887 from the 5′ end of the genomic DI RNA, was used for cDNA synthesis and oligonucleotide 52 was used as the second primer. For amplification of helper virus genomic RNA, oligonucleotide 10010 (5′-TGCAGGAGTACTTACCCT-3′), which binds to positive-strand MHV RNA at nucleotides 901 to 918 from the 5′ end, was used for cDNA synthesis. The MHV DI RNAs used in this study did not contain a binding site for this oligonucleotide.

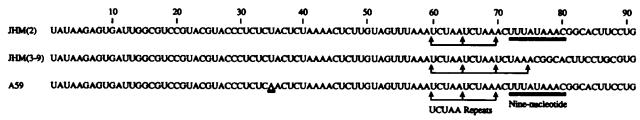

FIG. 2. Sequence of the 5′-end regions of JHM(2), JHM(3-9), and A59. The nine-nucleotide sequence (described in the text) is underlined by bold lines. Locations of UCUAA repeats are also shown. The divergent A nucleotide at position 35 of A59 is underlined.

Oligonucleotide 52 was used as a second primer. The conditions used for amplification of genomic DI RNA and helper virus genomic RNA were the same as those used for RT-PCR of subgenomic DI RNA.

Direct sequencing of the RT-PCR product. The PCR products were separated by agarose gel electrophoresis and recovered from the gel slices with GeneClean II (Bio 101, La Jolla, Calif.). Direct sequencing of the PCR product was performed as described previously (7, 36). For sequencing of RT-PCR products of genomic DI RNA and helper virus genomic RNA, oligonucleotide 55 (5′-GCTGGCGCCGAATGGACACG-3′), which binds to positive-strand helper virus genomic RNA at nucleotides 170 to 189 from the 5′ end, was used as a sequence primer. As the sequence primer of the subgenomic DI RNA-specific RT-PCR, oligonucleotide 2165 (5′-ATTTCGGGTT ACCGAGCTTTTGGG-3′), which binds to positive-strand subgenomic DI RNA at nucleotides 400 to 423 from the 3′ end, was used.

RESULTS

The major reasons for using DI cDNAs in the present study were that helper virus-derived RNAs and DI RNA-derived RNAs are easily separable by agarose gel electrophoresis and that DI sequences are routinely manipulated by standard molecular biological techniques. Because of the lack of a coronavirus infectious cDNA clone, there is no way to mutate MHV genomic sequences directly.

Effect of the helper virus genomic leader sequence on subgenomic DI RNA transcription. To determine whether the leader sequence has a *trans*-acting property, we monitored the association of the infectious MHV genomic leader sequence with subgenomic DI RNA transcripts. For the first set of experiments, two naturally occurring MHV JHM helper virus variants and two specially constructed DI cDNAs were used. These experiments were designed such that the leader sequence of the genomic DI RNA was inactive for transcription of the monitored subgenomic DI RNA. Transcription could only occur if the helper virus leader was used for subgenomic DI RNA transcription.

One helper virus, JHM(2), contained two UCUAA repeats in the 3′ region of the genomic leader sequence (22, 33). The other helper virus, JHM(3-9), had three UCUAA repeats, but downstream of the repeats it lacked nine nucleotides (UUUAUAAAC) (Fig. 2). Analysis of the JHM(2) and JHM(3-9) intracellular RNA species showed that mRNA 2-1, which encodes the HE gene (33), was made from JHM(2) and that neither mRNA 2-1 nor mRNA 2 was detected from JHM(3-9) (Fig. 3). Regulation of mRNA 2-1 synthesis is still not completely understood; however, we do know that when genomic leader RNA has two UCUAA repeats, mRNA 2-1 is synthesized and when three repeats are present in the genomic leader, only a trace amount of mRNA 2-1 is synthesized (20, 33). Analysis of JHM(3-9) mRNA suggested that the presence of the nine nucleotides may be crucial for the synthesis of

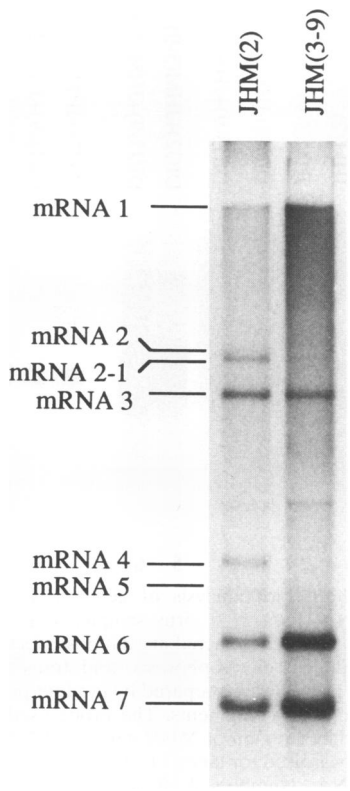

FIG. 3. Electrophoretic analysis of the virus-specific intracellular RNAs of JHM variants JHM(2) and JHM(3-9). ^{32}P-labeled intracellular viral RNAs were denatured with glyoxal and separated by electrophoresis on a 1% agarose gel.

mRNA 2-1. This speculation was consistent with the analysis of another MHV variant, JHM-2c, which also lacks the nine nucleotides and does not produce a detectable level of mRNA 2-1 (24). In addition to mRNA 2-1, only a trace amount of mRNA 4 was detected in JHM(3-9)-infected cells (Fig. 3). It is not known why the amount of mRNA 4 from JHM(3-9) was lower than that from JHM(2). Both viruses synthesized small amounts of mRNA 5, as demonstrated by Northern blot analysis (Fig. 4).

Two MHV JHM-derived DI cDNAs, DI(J2) and DI(J3-9), were constructed (Fig. 1). These DI cDNAs had similar structures and contained the same insertion, which was the intergenic region preceding the HE protein gene. MHV mRNA 2-1, which is synthesized from this intergenic region, is a functional mRNA with a coronavirus-specific mRNA structure (33). DI(J2) had two UCUAA repeats at the 3′ region of the leader sequence. DI(J3-9) differed in that it carried three UCUAA repeats and lacked the nine nucleotides from the 3′ region of the leader sequence. Except that both in vitro-synthesized DI RNAs contained one additional G residue at the very 5′ end (21), the leader sequences of JHM(2) and DI(J2) and those of JHM(3-9) and DI(J3-9) were the same. Had efficient mRNA 2-1 transcription occurred only when the leader sequence with two UCUAA repeats was used, then it was expected that the inserted intergenic region in the DI RNAs was transcriptionally functional only when a leader sequence with two UCUAA repeats was available for subgenomic DI RNA transcription. The intergenic region inserted in the DI RNAs was derived from a naturally occurring intergenic region from mRNA 2-1 (33), so that subgenomic DI

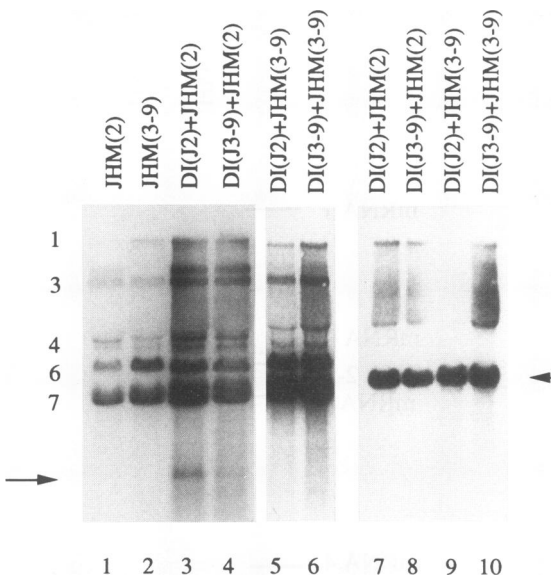

FIG. 4. Northern blot analysis of genomic DI RNA and subgenomic DI RNA. Passage 0 virus samples were used as inocula. Intracellular RNAs were extracted at 10 h postinfection, separated by 1% formaldehyde gel electrophoresis, and transferred to a nylon membrane. The probes were prepared by random-primed ^{32}P labeling of MHV-specific cDNA fragments. The probe used for lanes 1 to 6 corresponds to the 3′ region of MHV genomic RNA. An MHV gene 1-derived probe was used for lanes 7 to 10 to detect only mRNA 1 and genomic DI RNA. Numbers 1, 3, 4, 6, and 7 represent major MHV-specific mRNA species. The arrowhead and arrow indicate genomic and subgenomic DI RNAs, respectively.

RNA was most likely synthesized by MHV-specific transcription mechanisms.

If a helper virus-derived leader sequence is used for subgenomic DI RNA transcription, then subgenomic DI RNA should be transcribed in DI(J3-9)-replicating and JHM(2)-infected cells but not in DI(J3-9)-replicating and JHM(3-9)-infected cells. To examine whether the helper virus genomic leader sequence influences subgenomic DI RNA transcription, synthesis of subgenomic DI RNA was examined in cells infected with JHM(2) or JHM(3-9). The in vitro-synthesized DI RNAs were transfected by lipofection into monolayers of DBT cells that had been infected with JHM(2) or JHM(3-9) 1 h prior to transfection (21). After incubation of virus-infected cells at 37°C for 16 h, the culture fluid was harvested; this sample was named passage 0. This passage 0 virus sample was used as an inoculum for analysis of intracellular RNA species. Virus-specific intracellular RNA was extracted at 10 h postinfection and analyzed by Northern blotting with a probe which specifically hybridizes with all MHV RNAs and a probe which specifically hybridizes with genomic DI RNA and mRNA 1 (Fig. 4). The amount of the negative-strand coronavirus RNA species is much less than that of the positive-strand coronavirus RNA species (29); therefore, the signal in this Northern blot analysis represented mostly positive-strand RNA species. Replication of both 2-kb-long genomic DI RNAs was observed in cells infected with both helper viruses (Fig. 4). Radioactive smears apparent in lanes 7 to 10 probably represent partially degraded mRNA 1 from helper virus. Synthesis of 0.6-kb-long subgenomic DI RNAs derived from DI(J2) and DI(J3-9) was observed only in JHM(2)-infected cells, whereas no subgenomic DI RNA synthesis was observed in JHM(3-9)-infected cells (Fig. 4). The molar ratio of subgenomic DI RNA to genomic DI RNA of DI(J2) was approximately 0.1, and it was consistently higher than that of DI(J3-9) in repeated experiments. More subgenomic RNA is transcribed from a gene 7-intergenic region than from an HE gene-intergenic region (33; Fig. 3). This was also true in the DI system; the molar ratio of DI(J2) subgenomic DI RNA to DI(J2) genomic RNA was lower than the ratios of subgenomic DI RNAs initiated from inserted intergenic regions preceding gene 7 to their DI genomes (18). This difference probably reflected the differences in transcriptional activity of these two intergenic regions. Faint signals which migrated faster than subgenomic DI RNA can be seen in Fig. 4, lanes 5 and 6; these were not consistently detected, and their origin is not clear.

To confirm the Northern blot data, subgenomic DI RNA synthesis was further examined by RT-PCR in which the 5′ region of subgenomic DI RNAs was amplified. As shown in Fig. 5A, cells infected with JHM(2) yielded the 0.6-kb-long subgenomic DI RNA-specific PCR products expected. The 1.7-kb-long band represented the RT-PCR product of mRNA 7. The 2-kb-long genomic DI RNA-specific PCR products were not detected under this RT-PCR condition. In repeated experiments, a subgenomic DI RNA-specific RT-PCR product was not observed in DI(J3-9)-replicating, JHM(3-9)-infected cells. Minute amounts of a subgenomic DI RNA-specific RT-PCR product were sometimes observed in DI(J2)-replicating, JHM(3-9)-infected cells, whereas the appearance of this RT-PCR product was sporadic (data not shown). To determine whether the inserted intergenic region was used for transcription, the PCR product of DI(J3-9)-derived subgenomic DI RNA was sequenced directly (Fig. 5B). The sequence analysis demonstrated that the leader-body fusion site of subgenomic DI RNA was the same as that of mRNA 2-1 (33). We confirmed the direct sequencing data by subcloning and sequencing the RT-PCR products derived from DI(J2) and DI(J3-9) subgenomic DI RNAs. The same sequence was obtained after analysis of five independent clones from each PCR product.

The leader sequences of the genomic DI RNA and helper virus genomic RNA from passage 0 virus-infected cells were determined. This analysis was necessary because the leader sequence of DI RNA switches to that of helper virus at a high frequency in certain combinations of helper virus and DI RNAs (21). The 5′ region sequences of helper virus genomic RNAs and genomic DI RNAs were determined from helper virus-specific and genomic DI RNA-specific RT-PCR products. Oligonucleotide 10010 binds to helper virus genomic RNA but not to genomic DI RNA (Fig. 1) and was used for amplification of helper virus RNA in combination with oligonucleotide 52, which binds to the leader sequence. This reaction produced the expected 0.9-kb RT-PCR product (Fig. 6A). For RT-PCR analysis of genomic DI RNA, oligonucleotide 1568, which binds 0.88 kb from the 5′ end of genomic DI RNA, was used in combination with oligonucleotide 52. As shown in Fig. 6B, the expected 0.9-kb-long genomic DI RNA-specific RT-PCR products were made. Oligonucleotide 1568 binds to the helper virus genome 3.1 kb from the 5′ end (Fig. 1); however, a helper virus-specific RT-PCR product was not detected by using this oligonucleotide and oligonucleotide 52. Probably the RT-PCR condition was not optimal for efficient amplification of the 3.1-kb-long helper virus-specific RT-PCR product. Direct sequencing of RT-PCR products demonstrated that all of the helper virus genomic RNAs and most of the genomic DI RNAs maintained their original leader sequences in passage 0 virus-infected cells (Fig. 6C). The genomic DI RNA leader sequence extracted from cells in-

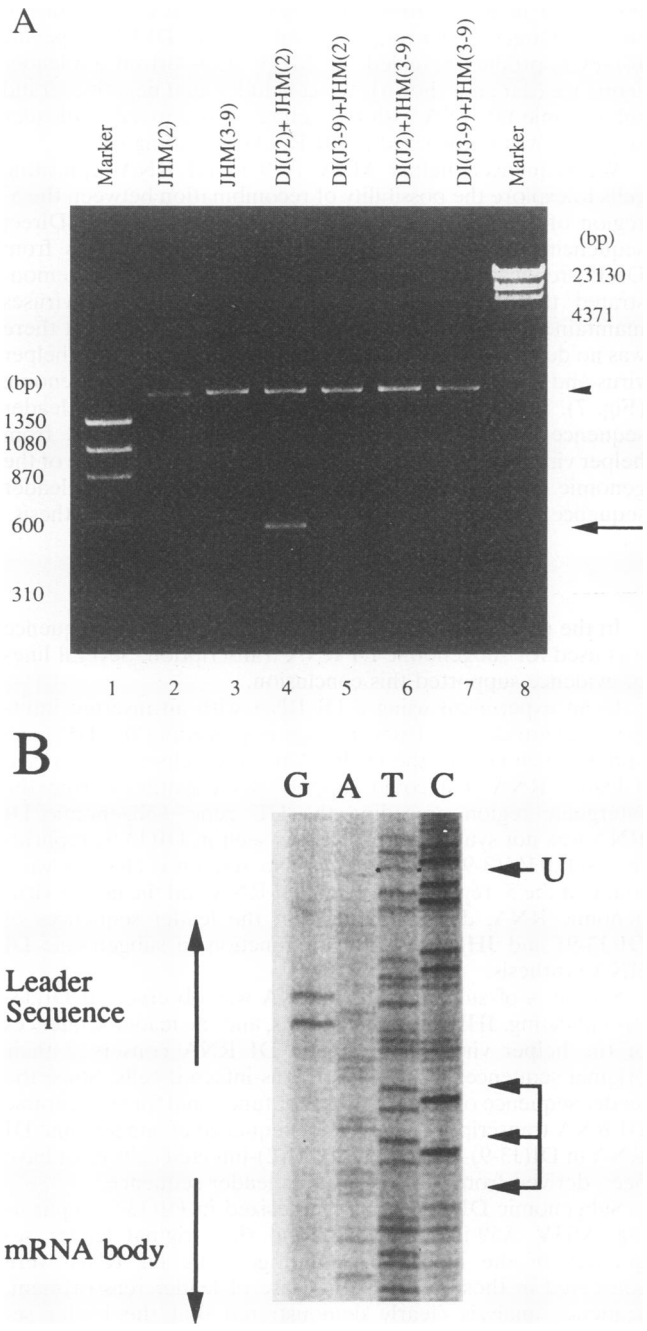

FIG. 5. Analyses of subgenomic DI RNA-specific RT-PCR products. (A) Agarose gel electrophoresis of RT-PCR products amplified from DI RNA-replicating cells. Oligonucleotides 130 and 52 were used for RT-PCR. The subgenomic DI RNA-specific PCR products are shown by the arrow. The arrowhead shows the mRNA 7-specific PCR products. The size markers are *Hae*III-digested ϕX174 DNA (lane 1) and *Hin*dIII-digested lambda DNA (lane 8) (New England BioLabs). DNA was stained with ethidium bromide. (B) Direct sequencing of the subgenomic DI RNA-specific RT-PCR products synthesized from DI(J3-9)-replicating, JHM(2)-infected cells. Oligonucleotide 2165 was used a sequence primer. The UCUAA repeats are shown by arrows. The leader sequences of DI RNAs and JHM contained a U residue at position 35, which is shown by an arrow. MHV A59 contains an A residue at the same position (see Fig. 2 and 9).

fected with the virus sample derived from DI(J2)-transfected, JHM(3-9)-infected cells was heterogeneous at the leader sequence; the major sequence contained three UCUAA repeats and the nine-nucleotide deletion, and the minor sequence had the original sequence with two UCUAA repeats and the nine nucleotides present (Fig. 6C and 7). This indicated that the leader sequence of most of the DI(J2) genomic DI RNA in JHM(3-9)-infected cells was switched to the helper virus-derived leader sequence.

DI(J3-9) subgenomic DI RNA synthesis occurred in JHM(2) helper virus-infected cells, and no subgenomic DI RNA synthesis occurred in DI(J3-9)-replicating, JHM(3-9)-replicating cells. This strongly indicated that the JHM(2)-derived leader sequence functioned in DI(J3-9) subgenomic DI RNA transcription.

Helper virus-derived leader sequence was used for subgenomic DI RNA transcription. To demonstrate that the helper virus-derived leader sequence was indeed used for subgenomic DI RNA transcription, MHV strain A59 was used as a "marked" helper virus in the next experiment. The genomic leader sequences of JHM(2) and MHV A59 carry two UCUAA repeats and the nine nucleotides (21). The leader sequences of both viruses are nearly identical, except that JHM has a U residue at nucleotide 35, where MHV A59 has an A (Fig. 2) (21). This nucleotide difference was used as a marker for identification of the origin of the subgenomic DI RNA leader sequence.

In vitro-synthesized DI(J2) and DI(J3-9) RNAs were transfected into MHV A59-infected cells, and passage 0 viruses were prepared. Northern blot analysis of MHV-specific RNAs from passage 0 virus-infected cells revealed that MHV A59 supported replication and transcription of DI RNAs (Fig. 8). The molar ratio of subgenomic DI RNA to genomic DI RNA of DI(J2) was higher than that of DI(J3-9), and this result was reproducible. By densitometric analysis of autoradiograms, the molar ratios of subgenomic DI RNA to genomic DI RNA in DI(J2) and DI(J3-9) were, on average, 0.3 and 0.2, respectively.

To measure RNA-RNA recombination effects in these experiments, genomic DI RNA-specific RT-PCR products were generated for use in sequence checking of the leader. Direct sequencing of the RT-PCR products showed no change in the leader sequence of DI(J3-9), whereas the DI(J2) leader sequence switched to that of MHV A59 (Fig. 7). This result was consistent with our previous report that DI RNA leader sequences with the nine nucleotides switch leaders and those without the nine nucleotides do not switch (21).

Direct sequencing of the subgenomic DI RNA-specific RT-PCR products revealed that both subgenomic DI RNAs contained the MHV A59-derived leader sequence (Fig. 9). To confirm the direct sequencing data, RT-PCR products were subcloned into a plasmid vector and sequenced. Sequencing of 10 clones from each PCR product demonstrated, without exception, that subgenomic DI RNAs contained the MHV A59-derived leader sequence.

Subgenomic-size negative-strand RNAs with an antileader sequence are present in coronavirus-infected cells (31). We looked at negative-strand RNA to confirm that DI and genomic RNAs share the same transcription mechanism. MHV-specific cDNAs were synthesized from total intracellular RNA from DI(J3-9)-replicating, MHV A59-infected cells by using oligonucleotide 52, which hybridizes to the antileader sequence, as a primer. Those cDNA products were incubated with oligonucleotide 130, which binds to the 3′ ends of all positive-strand MHV RNA species, in an RT-PCR. The products included a 0.6-kb-long product which was predicted to

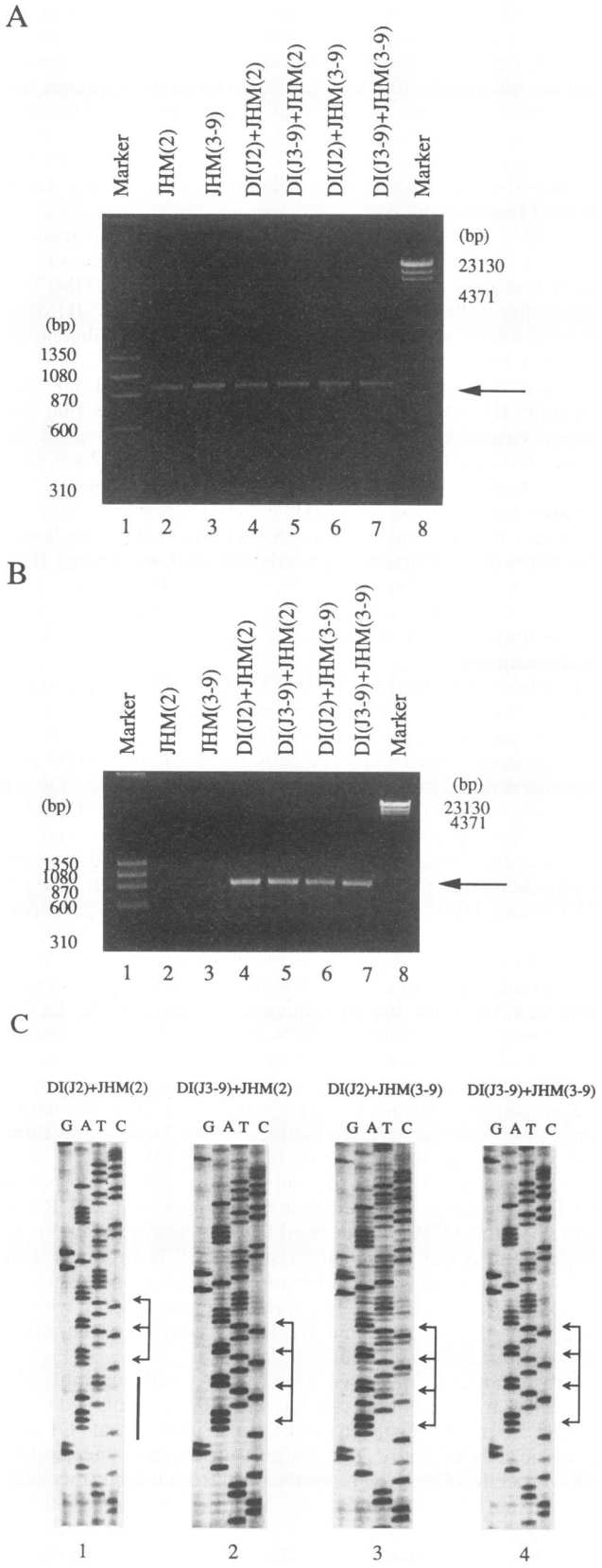

FIG. 6. Analyses of helper virus genomic RNA-specific RT-PCR products (A) and genomic DI RNA-specific RT-PCR products (B and C). (A) Agarose gel electrophoresis of helper virus-specific RT-PCR amplified from DI RNA-replicating cells. Oligonucleotides 10010 and 52 were used for RT-PCR. The helper virus-specific PCR products are shown by the arrow. The size markers are *Hae*III-digested ϕX174 DNA (lane 1) and *Hin*dIII-digested lambda DNA (lane 8) (New England BioLabs). DNA was stained with ethidium bromide. (B) Agarose gel electrophoresis of genomic DI RNA-specific RT-PCR amplified from DI RNA-replicating cells. Oligonucleotides 1568 and 52 were used for RT-PCR. The genomic DI RNA-specific PCR products are shown by the arrow. (C) Direct sequencing of the genomic DI RNA-specific PCR products. Oligonucleotide 55 was used as a sequence primer. The UCUAA repeats are shown by arrows. The nine-nucleotide sequence is shown by the bold line.

come from the subgenomic DI RNA. This 0.6-kb product was not found in RNA from MHV A59-infected cells (data not shown). Direct sequencing of the subgenomic DI RNA-specific RT-PCR product revealed the MHV A59-derived antileader sequence (data not shown). We concluded that negative-strand subgenomic DI RNA with the helper virus-derived antileader sequence was synthesized in DI RNA-replicating cells.

We sequenced helper MHV A59 in DI RNA-replicating cells to explore the possibility of recombination between the 5′ region of the helper virus genome and the DI RNA. Direct sequencing of helper virus-specific RT-PCR products from DI(J2)-replicating cells and DI(J3-9)-replicating cells demonstrated that the leader sequences of both helper viruses maintained their original sequences, demonstrating that there was no detectable level of RNA recombination between helper virus and DI RNA at the 5′ region of the helper virus genome (Fig. 7). These analyses clearly demonstrated that the leader sequence on DI(J3-9) subgenomic DI RNA derived from helper virus MHV A59 but not from the leader sequence of the genomic DI RNA. We concluded that the helper virus leader sequence worked in *trans* for subgenomic DI RNA synthesis.

DISCUSSION

In the present study, a helper virus-derived leader sequence was used for subgenomic DI RNA transcription. Several lines of evidence supported this conclusion.

In an experiment using a DI RNA with an inserted intergenic region derived from the region preceding the HE gene and in which neither the DI RNA nor the helper virus carried a leader RNA that could support RNA synthesis from the intergenic region preceding the HE gene, subgenomic DI RNA was not synthesized. This was seen in DI(J3-9)-replicating and JHM(3-9)-infected cells. No sequence changes were found at the 5′ region of genomic DI RNA and the helper virus genomic RNA, demonstrating that the leader sequences of DI(J3-9) and JHM(3-9) did not function in subgenomic DI RNA synthesis.

Synthesis of subgenomic DI RNA was observed in DI(J3-9)-replicating, JHM(2)-infected cells, and the leader sequences of the helper virus and genomic DI RNA conserved their original sequences in passage 0 virus-infected cells. Since the leader sequence of DI(J3-9) was not functional for subgenomic DI RNA transcription, the leader sequence of subgenomic DI RNA in DI(J3-9)-replicating, JHM(2)-infected cells must have been derived from the helper virus leader sequence.

Subgenomic DI RNA was synthesized in DI(J3-9)-replicating, MHV A59-infected cells, and the original leader sequences of the helper virus and genomic DI RNA were conserved in those cells. In this case of leader reassortment, sequence analysis clearly demonstrated that the leader sequence of the subgenomic DI RNA was derived from the

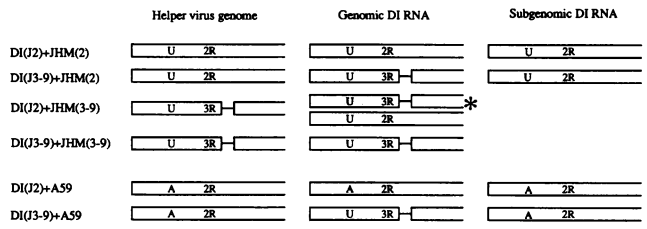

FIG. 7. Diagram of the 5′-end sequences of helper virus genomic RNAs, genomic DI RNAs, and subgenomic DI RNAs from passage 0 virus-infected cells. The sequences of subgenomic DI RNAs were obtained by direct PCR sequencing and sequencing of cloned PCR products. All of the other sequence data come from direct sequencing of RT-PCR products. 2R and 3R represent two and three repeats of the UCUAA sequence, respectively. The deletion of the nine-nucleotide sequence is shown as a thin line. Letters U and A represent nucleotide 35 of each RNA. Genomic DI RNA obtained from cells infected with the virus sample derived from DI(J2)-transfected, JHM(3-9)-infected cells demonstrated sequence heterogeneity. The major RNA species is shown by the asterisk. The shaded areas represent genomic DI RNAs that underwent leader switching.

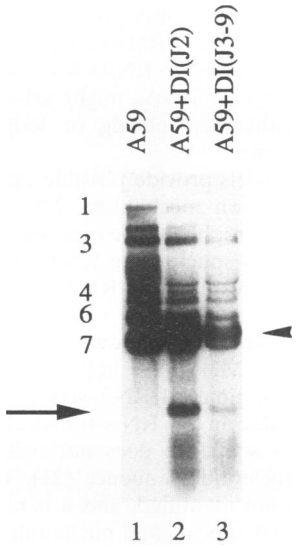

FIG. 8. Northern blot analysis of genomic and subgenomic DI RNAs. Passage 0 virus samples were used as inocula. Intracellular RNAs were extracted at 7 h postinfection, separated by 1% formaldehyde gel electrophoresis, and transferred to a nylon membrane. The probe was prepared by random-primed ^{32}P labeling of the MHV-specific cDNA fragment corresponding to the 3′ region of MHV genomic RNA. Numbers 1, 3, 4, 6, and 7 represent major MHV A59-specific mRNA species. The arrowhead and arrow indicate genomic and subgenomic DI RNAs, respectively.

helper virus. MHV A59 helper virus leader RNA functioned in *trans* in the subgenomic DI RNA transcription of a DI RNA derived from MHV JHM.

Analyses of subgenomic DI RNAs revealed that the subgenomic DI RNAs were most probably synthesized by using the MHV transcription mechanism. The inserted intergenic region used in the present study was derived from that preceding the HE gene. MHV mRNA 2-1, which is synthesized from this intergenic region, encodes the MHV HE protein, and this mRNA has a characteristic coronavirus mRNA structure. Northern blot analysis clearly demonstrated synthesis of subgenomic DI RNA (Fig. 4 and 8). The molar ratio of subgenomic DI(J2) RNA to genomic DI(J2) RNA in A59-infected cells was about 0.3, and the ratio of subgenomic DI RNA to its genomic DI RNA was similar to that of another DI RNA, which has one complete UCUAAAC intergenic consensus sequence (7). Subgenomic DI RNA in JHM(2)-infected cells has the same leader-body fusion site as JHM(2) mRNA 2-1 (33). Correspondingly, the negative-strand subgenomic DI RNA contained the antileader sequence present in DI RNA-replicating cells. These findings indicate that subgenomic DI RNAs had characteristic coronavirus mRNA properties. It is highly unlikely that all subgenomic DI RNA molecules were generated by some other mechanisms, e.g., aberrant homologous recombination between helper virus-derived leader RNA and the DI RNA sequence (10). Therefore, the data from the present study revealed that, indeed, the leader sequence functions in *trans* for coronavirus transcription.

The *trans*-acting property of coronavirus leader RNA indicated that coronavirus transcription involves discontinuous transcription. Discontinuous transcription may take place during primary or secondary transcription. If discontinuous transcription occurs during primary transcription, then it may include a leader RNA-primed or *trans*-splicing type of transcription. Should discontinuous transcription happen during secondary transcription, one possible mechanism is a modified leader-primed transcription; free leader RNAs (4) might prime subgenomic RNA synthesis on the subgenomic-size, negative-strand template RNAs made during primary transcription that would lack the antileader sequence. Subgenomic-size, negative-strand RNA containing an antileader sequence is seen in coronavirus-infected cells (31, 32). Possibly when leader RNA primes secondary transcription, the antileader sequence of subgenomic-size, negative-strand RNA is acquired: either by a replicon-type, subgenomic-RNA amplification (32) or during synthesis of subgenomic negative-strand RNA on the subgenomic mRNA template. If subgenomic negative-strand RNA is made on a subgenomic mRNA template, then these negative-strand RNAs may be dead-end products of transcription (6). Another way that discontinuous transcription could

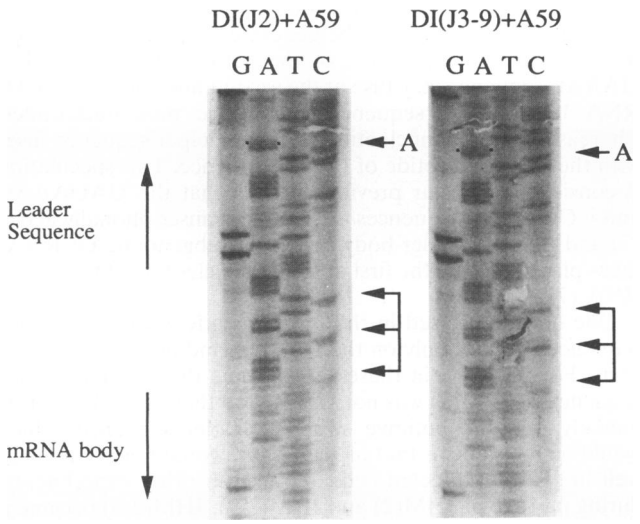

FIG. 9. Direct sequencing of the subgenomic DI RNA-specific RT-PCR products synthesized from DI(J2)-replicating, MHV A59-infected cells and (J3-9)-replicating, MHV A59-infected cells. Oligonucleotide 2165 was used as a sequence primer. The UCUAA repeats are shown by arrows. The leader sequence of subgenomic DI RNA contained an MHV A59-derived A nucleotide at position 35.

happen during secondary transcription is if the subgenomic-size, negative-strand template RNAs containing the antileader sequence function as template RNAs for leader-primed transcription. These template RNAs might arise during primary transcription by either *cis* splicing or looping out of the positive-strand genome.

Many different models provide possible explanations of the coronavirus transcription mechanism. The data we present here exclude a faithful replicon type of secondary transcription which follows either *cis*-splicing-like RNA synthesis or looping out of the genomic-size template RNA during primary transcription.

The leader sequence of DI RNA with two UCUAA repeats is switched to that of MHV A59 helper virus at a very high frequency, and the original DI RNA-specific leader sequence is no longer detectable in DI RNA-transfected cells (21). In contrast, the leader sequence does not switch in DI RNAs without the nine-nucleotide sequence (21). The exact site of leader switching is not identified, and it is mapped anywhere between the UCUAA repeats and nucleotide 110 (21). These findings were confirmed by DI(J2) leader sequence switching to that of MHV A59 and by lack of switching of the DI(J3-9) leader sequence to that of helper virus A59. Unexpectedly, we found that the DI(J2) leader sequence also switched to that of a JHM variant, JHM(3-9). Although the leader switching mechanism is unknown, the leader switching event on DI(J2) RNA in JHM(3-9)-infected cells may indicate that the same mechanism which regulates the subgenomic mRNA leader-body fusion event also functions in leader switching. In other words, the leader switching may be the result of a leader-body fusion event which occurs at the 5′ region of the genomic DI RNA. The UAUAAAC sequence within the nine-nucleotide sequence is very similar to the UCUAAAC sequence in the last leader sequence UCUAA repeat. UCUAAAC is the transcription consensus sequence, which is required for subgenomic mRNA transcription (7). Direct sequencing analysis of genomic DI RNA derived from DI(J2)-replicating, JHM(3-9)-infected cells demonstrated that the major sequence at the 3′ region of the leader sequence was 5′-UAGUUUAAAUC UAAUCUAAUC/AUAAACGG-3′ (Fig. 6C, panel 3). This leader RNA sequence may be generated by two simultaneous fusions: the first nucleotide of the helper virus-derived UC UAAAC sequence may fuse at the second nucleotide of the DI RNA UAUAAAC sequence, within the nine nucleotides, whereas the second nucleotide from the helper sequence fuses with the third nucleotide of the DI sequence. This speculation is consistent with our previous finding that the UAUAAAC and UCUAAAC sequences are equally transcriptionally active (7) and that the leader-body fusion on subgenomic DI RNAs takes place at either the first or second nucleotide of the leader RNA (7).

One question raised in the present study is why the leader sequence switches only on the DI RNA and not on the helper virus. For instance, in these experiments the genomic leader sequence of JHM(2) was not switched to that of DI(J3-9). It is unlikely that the putative switched leader sequence, which would be the same as that of JHM(3-9), would not accumulate well in JHM(2)-infected cells, because in other experiments, during passage of JHM(2) and JHM(3-9), JHM(3-9) became a major population after several passages (unpublished data), suggesting that JHM(3-9) has some advantage. Another unlikely explanation for failure to detect leader switching on helper virus genomic RNA is that maybe only a small population of cells were infected with DI particles. Our preliminary data indicated otherwise; a high percentage of passage 0 virus-infected cells were coinfected with DI particles and the helper virus (unpublished data).

A more likely explanation for the lack of leader switching on the helper virus leader may be that free leaders are not synthesized from DI RNA. If this is the case and free-leader RNA is used for subgenomic mRNA transcription, then the DI RNA-derived leader RNA may not be used for subgenomic DI RNA transcription; in a combination with DI(J2) and the JHM(3-9) helper virus, we examined whether the leader RNA of DI RNA was used for subgenomic DI RNA transcription. Unfortunately, the leader RNA of DI(J2) was switched to that of JHM(3-9) at a high frequency; therefore, this question remains unanswered. Minute amounts of subgenomic DI RNA-specific RT-PCR products were sometimes found in cells infected with the virus sample harvested from DI(J2)-transfected, JHM(3-9)-infected cells. Some of the DI(J2)-derived DI RNAs maintained their original sequences (Fig. 6C and 7). Perhaps DI(J2) leader RNA was used for subgenomic DI RNA transcription at low efficiency. Alternatively, it is possible that a minor population of the JHM(3-9) leader sequence was switched to that of DI(J2) DI RNA by homologous RNA recombination between DI RNA and helper virus genomic RNA and a genomic leader RNA derived from the recombinant helper virus was used for subgenomic DI RNA transcription.

The molar ratio of DI(J2) subgenomic DI RNA to genomic DI RNA was consistently higher than that of DI(J3-9) in JHM(2)-infected cells. The result obtained with MHV A59-infected cells was similar. Genomic DI RNAs which contained two UCUAA repeats supported more efficient subgenomic DI RNA transcription than did those with three UCUAA repeats and the nine-nucleotide deletion. This may indicate that the genomic DI RNA-specific leader sequence containing two UCUAA repeats was used for subgenomic DI RNA transcription in the *cis* orientation. We think that the higher level of subgenomic DI RNA transcribed from genomic DI RNA with two UCUAA repeats results from the use of both helper virus-derived and genomic DI RNA-derived leaders. Alternatively, this may suggest that during subgenomic DI RNA transcription, there is a step at which DI RNA leader RNA and the intergenic sequence interact intramolecularly and this interaction may be important for the utilization of helper virus-derived leader RNA for subgenomic DI RNA transcription. An intramolecular structure made by the leader RNA of DI(J2) and the intergenic region should differ from that made by the leader RNA of DI(J3-9) and the intergenic region. A structural difference may affect the efficiency of helper virus-derived leader RNA utilization.

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