JOURNAL OF VIROLOGY, June 1993, p. 3304–3311
0022-538X/93/063304-08$02.00/0

Vol. 67, No. 6

Effect of Intergenic Consensus Sequence Flanking Sequences on Coronavirus Transcription

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Received 14 December 1992/Accepted 8 March 1993

Insertion of a region, including the 18-nucleotide-long intergenic sequence between genes 6 and 7 of mouse hepatitis virus (MHV) genomic RNA, into an MHV defective interfering (DI) RNA leads to transcription of subgenomic DI RNA in helper virus-infected cells (S. Makino, M. Joo, and J. K. Makino, J. Virol. 66:6031–6041, 1991). In this study, the subgenomic DI RNA system was used to determine how sequences flanking the intergenic region affect MHV RNA transcription and to identify the minimum intergenic sequence required for MHV transcription. DI cDNAs containing the intergenic region between genes 6 and 7, but with different lengths of upstream or downstream flanking sequences, were constructed. All DI cDNAs had an 18-nucleotide-long intergenic region that was identical to the 3′ region of the genomic leader sequence, which contains two UCUAA repeat sequences. These constructs included 0 to 1,440 nucleotides of upstream flanking sequence and 0 to 1,671 nucleotides of downstream flanking sequence. An analysis of intracellular genomic DI RNA and subgenomic DI RNA species revealed that there were no significant differences in the ratios of subgenomic to genomic DI RNA for any of the DI RNA constructs. DI cDNAs which lacked the intergenic region flanking sequences and contained a series of deletions within the 18-nucleotide-long intergenic sequence were constructed to determine the minimum sequence necessary for subgenomic DI RNA transcription. Small amounts of subgenomic DI RNA were synthesized from genomic DI RNAs with the intergenic consensus sequences UCUAAAC and GCUAAAC, whereas no subgenomic DI RNA transcription was observed from DI RNAs containing UCUAAAG and GCTAAAG sequences. These analyses demonstrated that the sequences flanking the intergenic sequence between genes 6 and 7 did not play a role in subgenomic DI RNA transcription regulation and that the UCUAAAC consensus sequence was sufficient for subgenomic DI RNA 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, 25). Seven to eight species of virus-specific subgenomic mRNAs are made in MHV-infected cells. These subgenomic mRNAs make up a 3′-coterminal nested set (12, 16). They are named, in decreasing order of size, mRNAs 1 through 7 (12, 16). Of these mRNA species, only mRNA 1 is efficiently packaged into MHV virions as a result of the presence of a packaging signal (4, 24, 36); the other mRNAs are not packaged (14, 21).

The 5′ end of the MHV genomic RNA contains a 72- to 77-nucleotide-long leader sequence (11, 13, 35). Within the 3′ region of the leader sequence there is a pentanucleotide sequence, UCUAA, that is repeated two to four times in different MHV strains (19, 22). The MHV-specific genes are downstream from the leader. The genes are separated from one another by a special short stretch of sequence, the intergenic sequence. Each intergenic sequence, located upstream of a gene essential for MHV replication, includes the unique consensus sequence of UCUAAAC or a very similar sequence (32). Each MHV mRNA species has a leader sequence which is identical to the 5′-end genomic leader sequence. The leader sequence is fused to the intergenic consensus sequence, which marks the start of the gene (32). The site where the leader fuses with the mRNA at the intergenic sequence includes a repeated pentanucleotide (UCUAA), and the number of repeats in each given mRNA varies (22).

It has been clearly demonstrated that 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, and the other is secondary transcription, for which subgenomic-size RNA serves as template (8, 18, 37). All of the activities necessary for MHV RNA synthesis are present continuously during the first 6 h of infection (8).

Several models have been proposed to explain how coronavirus synthesizes and accumulates subgenomic mRNA species. One is leader RNA-primed transcription. This model 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 (2, 10). In contrast, Sawicki and Sawicki (29) proposed that subgenomic negative-stranded RNAs are initially synthesized from input genomic RNA. Then, positive-stranded subgenomic RNA is synthesized from the subgenomic negative-stranded RNA. The leader sequence of the subgenomic RNA may be acquired during the initial subgenomic negative-stranded RNA synthesis or during subgenomic positive-stranded RNA synthesis (29). Another possible mechanism may be that leader RNA joins the subgenomic RNA body by a mechanism similar to RNA splicing (3, 13). Such splicing could take place during positive-stranded or negative-stranded RNA synthesis. On the basis of the observation that subgenomic negative-stranded RNAs containing the antileader sequence are present in coronavirus-infected cells (30), Sethna et al. proposed that subgenomic mRNA synthesis may be involved in the amplification of each subgenomic RNA species (31). This hypothesis is consistent with the observation that subgenomic replicative intermediate RNAs corresponding to each MHV subgenomic mRNA species are present in MHV-

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infected cells (29). It has also been proposed that only the negative-stranded RNA molecules are elongated on subgenomic positive-stranded RNA template (8). Another possibility is that subgenomic mRNA undergoes its amplification step at a reduced efficiency (8). None of these models has been exclusively proven or disproven.

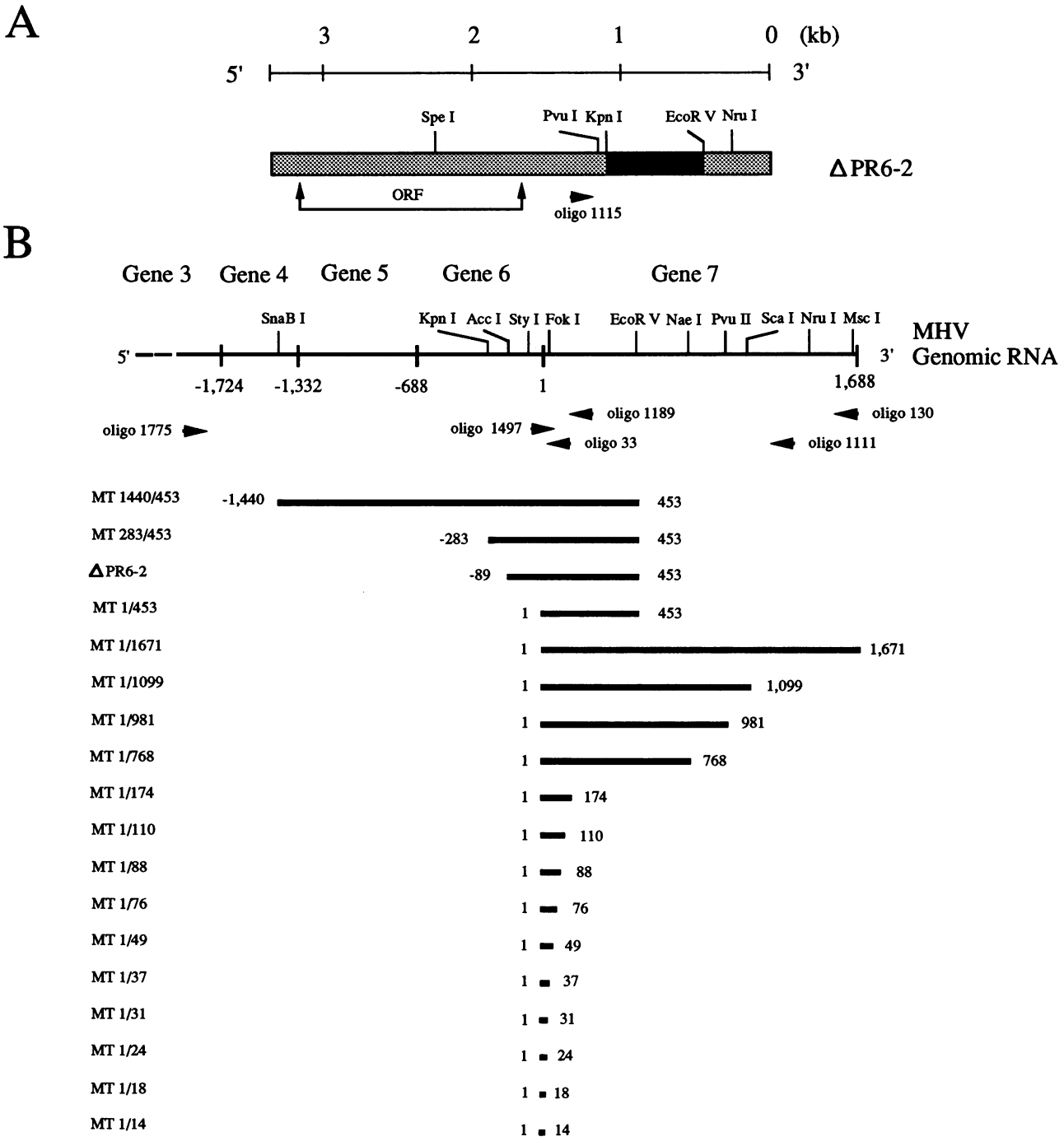
A system that exploits defective interfering (DI) RNAs of MHV for studying the mechanisms of coronavirus mRNA transcription has been established (18). In this system, a complete MHV DI cDNA clone containing an inserted intergenic region, derived from between genes 6 and 7 of the genome, was constructed. Replication of genomic DI RNA as well as transcription of subgenomic DI RNA was observed after transfection of in vitro-synthesized DI RNA into MHV-infected cells. Analysis of a series of deletion mutations in the intergenic region demonstrated that the sequences flanking the consensus sequence of UCUAAAC affected the efficiency of subgenomic DI RNA transcription (18). Site-directed mutagenic analysis of the consensus sequence demonstrated that MHV transcription function is flexible enough to recognize mutated consensus sequences for subgenomic DI RNA transcription (9). Furthermore, the location of the leader-body fusion site on subgenomic DI RNA was identified (9).

A question left unanswered by these studies was why the ratio of subgenomic DI RNA to genomic DI RNA is essentially reversed compared with that of mRNA 7 to mRNA 1. It has been demonstrated that the amount of subgenomic DI RNA is about 80% of that of the genomic DI RNA, whereas the amount of mRNA 1 in MHV-infected cells is only 1.5% of that of mRNA 7 (16, 18). One possible explanation may be that all of these MHV DI RNAs lack enhancer elements for subgenomic DI RNA transcription. The effect of the sequences flanking the intergenic region on subgenomic DI RNA transcription was not previously examined. In this study, the role of the sequences flanking the gene 6-7 intergenic sequence in MHV RNA transcription was examined. It was found that the sequences flanking this intergenic region do not play a role in subgenomic DI RNA transcription. Furthermore, small amounts of subgenomic DI RNA were synthesized from genomic DI RNAs which had only the UCUAAAC consensus sequence as the inserted sequence. These finding are discussed in relation to the mechanism of coronavirus transcription.

MATERIALS AND METHODS

Viruses and cells. The plaque-cloned A59 strain of MHV (MHV-A59) (12) was used as a helper virus. The JHM strain of MHV (MHV-JHM) was used for the preparation of intracellular RNA species. Mouse DBT cells (7) were used for the growth of viruses.

DNA construction. The names of several oligonucleotides and the locations of their MHV binding sites are shown in Fig. 1. MHV DI cDNA clone ΔPR6-2 (8) was incubated with two oligonucleotides, 1497 (5′-CCGCATTGGTACCAATCT AA-3′), which contains a *Kpn*I site and binds to antigenomic-sense ΔPR6-2 at nucleotides 2329 to 2348 from the 5′ end, and 1111 (5′-GAGCCAGAAGGCTGCGAT-3′), which binds to genomic-sense ΔPR6-2 at nucleotides 357 to 374 from the 3′ end. The DNAs were incubated at 93°C for 30 s, 37°C for 45 s, and 72°C for 100 s in a polymerase chain reaction (PCR) buffer (0.05 M KCl, 0.01M Tris hydrochloride [pH 8.0], 0.0025 M $MgCl_2$, 0.01% gelatin, 0.17 mM [each] deoxynucleoside triphosphates, 5 U of *Taq* polymerase [Promega]) for 25 cycles. The 0.45-kb *Kpn*I-*Eco*RV fragment of the PCR product was inserted into the *Kpn*I-*Eco*RV site of ΔPR6-2, yielding MT 1/453. The 0.22-kb *Kpn*I-*Sty*I fragment of the MHV-JHM-specific cDNA clone, Am 20, which corresponds to the 5′ region of gene 7 through the 3′ region of gene 3 (31a), was ligated with the 0.52-kb *Sty*I-*Eco*RV fragment of ΔPR6-2 and digested with *Kpn*I and *Eco*RV. The 0.74-kb-long *Kpn*I-*Eco*RV fragment was inserted into the *Kpn*I-*Eco*RV fragment of ΔPR6-2, yielding MT 283/453. The 1.2-kb-long Am 8 (18) *Eco*RV-*Msc*I fragment was inserted at the *Eco*RV site of MT 1/453, yielding MT 1/1671. Total intracellular RNA was extracted from MHV-JHM-infected DBT cells at 7 h postinfection and mixed with random primer, and cDNA was synthesized according to the established procedure (27). The random-primed cDNA was incubated with oligonucleotide 1775 (5′-GGAGGACACCAGGACAG-3′), which hybridizes to the negative-stranded MHV-JHM sequence at nucleotides 3491 to 3507 from the 3′ end of MHV-JHM genomic RNA, and oligonucleotide 1189 (5′-GTTGGATATCTGCTTGG GC-3′), which contains an *Eco*RV site and binds to genomic-sense MHV-JHM at nucleotides 1506 to 1524 from the 3′ end under the same PCR conditions. The 1.6-kb-long *Sna*BI-*Eco*RV PCR fragment was inserted into the *Eco*RV site of MC 136-1 (18), yielding JW 2. The 0.9-kb-long *Kpn*I-*Nru*I MT 283/453 fragment was inserted into the *Kpn*I-*Nru*I large fragment of JW 2. The 4.1-kb *Pvu*I-*Pvu*I fragment of the resulting clone was inserted into the large *Pvu*I-*Pvu*I fragment of MT 283/453, yielding MT 1440/453. The 0.65-kb *Eco*RV-*Sca*I fragment of Am 8, the 0.5-kb *Eco*RV-*Pvu*II fragment of Am 8, and the 0.3-kb *Eco*RV-*Nae*I fragment of Am 8 were inserted into the *Eco*RV site of MT 1/453, yielding MT 1/1099, MT 1/981, and MT 1/768, respectively. MT 1/453 was incubated with two oligonucleotides, 1497 and 1189, under the PCR conditions described above. The 0.17-kb *Kpn*I-*Eco*RV fragment of the PCR product was inserted into the *Kpn*I-*Eco*RV site of MT 1/453, yielding MT 1/174. The same PCR product was digested with *Mbo*II, *Hin*fI, *Hin*pI, or *Msp*I and blunt ended. The *Kpn*I-*Mbo*II, *Kpn*I-*Hin*fI, *Kpn*I-*Hin*pI, and *Kpn*I-*Msp*I fragments were inserted into MT 1/453, yielding MT 1/110, MT 1/88, MT 1/76, and MT 1/49, respectively. The ΔPR6-2 was incubated with two oligonucleotides, 1115 (5′-TCTAGCACGTGG CACTA-3′), which binds to antigenomic-sense ΔPR6-2 at nucleotides 2272 to 2288 from the 5′ end, and 33 (5′-TTGCC CAGGAACAAAAGA-3′), which binds to genomic-sense ΔPR6-2 at nucleotides 3266 to 3283 from the 5′ end, under the PCR conditions described above. The 0.2-kb PCR product was digested with *Fok*I and blunt ended. After *Kpn*I digestion, the 0.14-kb *Kpn*I-*Fok*I fragment was inserted into the *Kpn*I-*Eco*RV site of MT 1/453. The resulting DNA clone was incubated with oligonucleotides 1497 and 130 (5′-TTC CAATTGGCCATGATCAA-3′), which bind to genomic-sense ΔPR6-2 at nucleotides 7 to 26 from the 3′ end, under the PCR conditions described above. The 0.2-kb *Kpn*I-*Nru*I fragment of the PCR product was inserted into the *Kpn*I-*Nru*I fragment of MT 1/453, yielding MT 1/37. MT 1/453 was incubated for PCR with oligonucleotides 1497 and 1615 (5′-CTTTCTGATATCCAAAAGACAT-3′). Oligonucleotide 1615 contains an *Eco*RV site and binds to genomic-sense MT 1/453 at nucleotides 3174 to 3195 from the 5′ end. The PCR product was digested with *Kpn*I and ligated with MT 1/453 which had been digested with *Kpn*I. After *Eco*RV digestion of the DNA followed by self-ligation, MT 1/31 was obtained. The same procedure was used to construct MT1/24, MT 1/18, and MT 1/14, using oligonucleotides 1496 (5′-CAG GAACGATATCCATCCTT-3′), 1678 (5′-CTTTCTGATAT


A
3
2
1
0 (kb)
5'
3'
Spe I
Pvu I Kpn I
EcoR V Nru I
ΔPR6-2
ORF
oligo 1115
B
Gene 3
Gene 4
Gene 5
Gene 6
Gene 7
SnaB I
Kpn I Acc I Sty I Fok I
EcoR V Nae I Pvu II Sca I Nru I Msc I
MHV
Genomic RNA
5'
3'
-1,724
-1,332
-688
1
1,688
oligo 1775
oligo 1497
oligo 1189
oligo 33
oligo 130
oligo 1111
MT 1440/453
-1,440
453
MT 283/453
-283
453
ΔPR6-2
-89
453
MT 1/453
1
453
MT 1/1671
1
1,671
MT 1/1099
1
1,099
MT 1/981
1
981
MT 1/768
1
768
MT 1/174
1
174
MT 1/110
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110
MT 1/88
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88
MT 1/76
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76
MT 1/49
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49
MT 1/37
1
37
MT 1/31
1
31
MT 1/24
1
24
MT 1/18
1
18
MT 1/14
1
14
C
-10
-5
1
5
10
15
20
25
Intergenic region
CCGUAUUGUUGAG AAUCUAAUCUAAACUUUA AGGAUGUCUU
Leader sequence
CUCUUGUAGUUUA AAUCUAAUCUAAACUUUA UAAACGGCAC

CTAAAGTTT-3'), and 1748 (5'-CTTTCTGATATCGTTTA GATT-3'), respectively, in place of oligonucleotide 1615. For the construction of all other DNA clones, a series of oligonucleotides containing the desired nucleotide deletion or degenerated bases at the target position was prepared, and PCR-based site-directed mutagenesis was performed (6). PCR products were then cloned into ΔPR6-2 or other MHV DI cDNA constructs prepared in this study. For each mutant, the entire region generated by insertion of the PCR product was sequenced to confirm the presence of the specific mutations and the absence of extraneous mutations. All MHV DI constructs used in this study had the same structure as ΔPR6-2 outside of inserted regions (Fig. 1).

RNA transcription and transfection. Plasmid DNAs were linearized by *Xba*I digestion, except MT 1440/453, which was digested by *Sma*I. DI RNAs were transcribed with T7 RNA polymerase as previously described (20). The lipofection procedure was used for RNA transfection as previously described (18).

Preparation of virus-specific intracellular RNA and Northern (RNA) blotting. Virus-specific RNAs in virus-infected cells were extracted as previously described (23). For each sample, 1.5 μg of intracellular RNA was denatured and electrophoresed through a 1, 1.2, or 1.7% agarose gel containing formaldehyde, and the separated RNA was blotted onto nylon filters as described previously (18). The nylon filter was soaked in a prehybridization buffer, and Northern blot hybridization was performed (4, 8). A gel-purified 0.25-kb *Nru*I-*Msc*I fragment from DFI-2 (24) was labeled by the random-priming procedure (27) and used as a probe. This probe corresponds to nucleotides 18 to 262 from the 3' end of the MHV DI cDNA.

RT-PCR. Reverse transcription (RT) of intracellular RNA from MHV-infected cells was done with avian myeloblastosis virus reverse transcriptase (Promega) and oligonucleotide 1111. The cDNA was amplified during 25 cycles of PCR with a second primer, oligonucleotide 78 (5'-AGCTTTACGTAC CCTCTCTACTCTAAAACTCTTGTAGTTT-3') (20), which hybridizes to negative-stranded MHV RNA at the leader sequence.

RESULTS

Role of the intergenic region flanking sequences in MHV subgenomic DI RNA synthesis. A series of DI cDNA clones was made to study how the upstream sequences flanking the intergenic region affected MHV subgenomic DI RNA synthesis. Each of these clones had an MHV-JHM gene 6-7 intergenic sequence with the same downstream flanking sequence; only the lengths of the upstream flanking sequence differed. An MHV DI cDNA clone, ΔPR6-2, with a complete intergenic region was used as the parent clone (8) (Fig. 1). In the present study, an A nucleotide located two nucleotides upstream of the first of the two intergenic UCUAA repeats is referred to as nucleotide 1 (Fig. 1C). The sequence from nucleotides 1 through 18 represents the intergenic region which has complete sequence homology with the 3' region of leader sequence (32). The ΔPR6-2 inserted sequence contained 89 nucleotides upstream and 453 nucleotides downstream of nucleotide 1 (Fig. 1B). The unique ΔPR6-2 *Kpn*I site shown in Fig. 1A was created during the DI cDNA construction (8, 18); it is located just upstream of the 89-nucleotide-long upstream flanking sequence (18). The mutant MHV DI cDNAs had various lengths of MHV sequence between the unique *Kpn*I and *Eco*RV sites of ΔPR6-2 (8, 18) (Fig. 1A and B). Most of the DI RNAs used were named according to their structure; e.g., MT 283/453 contained 283 nucleotides upstream and 453 nucleotides downstream of nucleotide 1 of the intergenic sequence, and MT 1/24 lacked an upstream intergenic flanking sequence and had a 24-nucleotide-long downstream sequence, including the 18-nucleotide-long intergenic sequence. This naming system was not used for ΔPR6-2 or for those mutants lacking intergenic region flanking sequences (see below).

The following four mutant DI cDNAs became the basis for this study: MT 1440/453, MT 283/453, ΔPR6-2, and MT 1/453. Comparisons of previously published MHV-JHM sequences (26, 33, 34) with those of genes 6, 5, and 4 of MT 1440/453 showed that genes 5b and 6 in this cDNA clone had several point mutations which did not affect the reading frame, whereas deletions disruptive to the reading frame were present in genes 4 and 5a (7a). The ability of MHV-JHM to replicate despite these disruptive mutations agreed with the recent observation that the proteins encoded in genes 4 and 5a are not necessary for MHV replication in tissue culture (38). The in vitro-synthesized DI RNAs transcribed from the four mutant DI cDNAs were transfected by lipofection into monolayers of DBT cells infected with MHV-A59 helper virus 1 h prior to transfection (18). After incubation of virus-infected cells at 37°C for 16 h, the culture fluid was harvested; this sample was named passage 0. To amplify DI particles (24), the passage 0 virus sample was further passaged, and the passage 1 virus sample was generated. This passage 1 sample was used as an inoculum for the analysis of intracellular RNA species. Virus-specific intracellular RNA was extracted at 7 h postinfection and analyzed by Northern blotting using a probe which specifically hybridizes with all MHV RNAs. The amount of negative-stranded coronavirus RNA species is much lower than that of positive-stranded coronavirus RNA species (28); therefore, the signal obtained from this Northern blot analysis represented mostly positive-stranded RNA species. As shown in Fig. 2A, the molar ratios of subgenomic DI RNA to genomic DI RNA were essentially the same for MT 1440/453, MT 283/453, ΔPR6-2, and MT 1/453. Because MT 1440/453 genomic DI RNA comigrated with 28S rRNA on the gels and sometimes appeared as a diffuse RNA band, RNA from this mutant was selected by oligo(dT) column chromatography prior to electrophoresis. The presence of subgenomic DI RNAs which were synthesized from inserted intergenic regions between genes 4 and 5 and genes 5 and 6

FIG. 1. Schematic diagram of the structure of ΔPR6-2 (A), structures of MHV-JHM genes 4 to 7 and other mutants (B), and sequences of the MHV intergenic region and the genomic leader sequence (C). All mutant DI RNAs had the same structure as ΔPR6-2, except that each mutant contained an inserted sequence, shown by a bold line in panel B, in place of the region represented by black box for ΔPR6-2 (A). The DI-specific open reading frame is labeled ORF in panel A (24). Restriction enzyme sites and oligonucleotides used for construction of the mutants are shown in panels A and B. The 18-nucleotide-long intergenic region homologous to the genomic leader sequence is underlined, and the first A nucleotide within the underlined sequence is defined as nucleotide 1. Leader sequence represents nucleotides 45 to 85 from the 5' end of MHV genomic RNA. The sequence upstream of the underlined sequence corresponds to the 3' region of the genomic leader sequence.

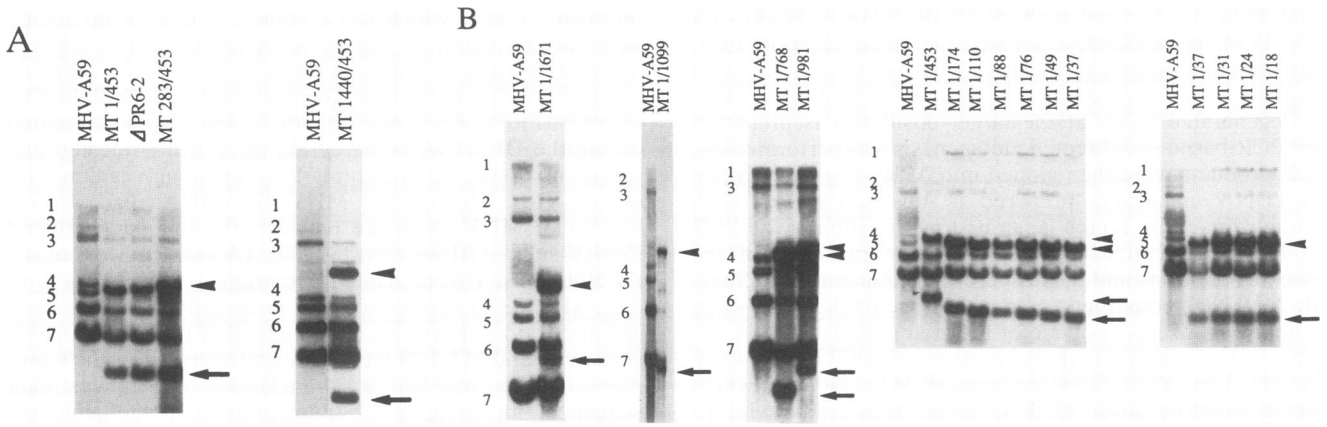

FIG. 2. Northern blot analysis of ΔPR6-2-derived mutant subgenomic DI RNAs. DI RNAs containing various lengths of upstream intergenic flanking sequences and those containing various lengths of downstream intergenic flanking sequences are shown in panels A and B, respectively. Passage 1 virus samples were used as inocula. Intracellular RNAs were extracted 7 h postinfection, separated by formaldehyde-agarose gel electrophoresis, and transferred to a nylon membrane. An agarose concentration of 1% was used for most of the analyses; the exceptions were that MT 1/1099 was applied to a 1.7% agarose gel, and MT 1/768 and MT 1/981 were applied to 1.5% gels. 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 to 7 indicate the MHV-A59-specific mRNA species. Arrowheads and arrows point to genomic DI RNA and subgenomic DI RNAs, respectively.

were also detected in MT 1440/453-replicating cells, using probes that specifically hybridize to genes 5 and 6 of MHV genomic RNA (data not shown). These data indicated that varying the length of the upstream flanking sequence from 0 to 1,440 nucleotides did not alter the ratio of subgenomic DI RNA to genomic DI RNA.

Next, the effect of the downstream intergenic flanking sequence on subgenomic DI RNA synthesis was examined. A series of MHV DI cDNAs, all lacking an upstream sequence but with various lengths of downstream sequence, including the 18-nucleotide-long intergenic region, was constructed (Fig. 1). Northern blot analysis of these mutants demonstrated that there were no significant differences in the molar ratios of genomic DI RNA to subgenomic DI RNA species (Fig. 2). This finding suggested that downstream sequences ranging from 18 to 1,671 nucleotides did not significantly enhance or suppress subgenomic DI RNA transcription.

Analysis of the minimum sequence requirement for subgenomic DI RNA synthesis. In an earlier study, deletion analysis of the 18-nucleotide-long intergenic sequence demonstrated that the sequences flanking the UCUAAAC consensus sequence affect the efficiency of subgenomic DI RNA transcription (18). In that study, the genomic DI RNA was PR6, which had flanking sequences of 89 nucleotides upstream and 453 nucleotides downstream of the 18-nucleotide consensus sequence 5′ end (18). The data presented here suggested that the intergenic region flanking sequences do not play a significant role in subgenomic DI RNA synthesis. Possibly the efficiency of subgenomic DI RNA transcription is solely determined by the sequence present within the intergenic region. To examine this possibility, a number of MHV DI cDNA clones were constructed. These completely lacked upstream and downstream intergenic region flanking sequences and had various deletions within the 18-nucleotide-long intergenic sequence (Fig. 3). Synthesis of subgenomic DI RNAs in passage 1 virus-infected cells was examined by Northern blot analysis (Fig. 3), and the molar ratio of subgenomic DI RNA to genomic DI RNA of each mutant was examined by densitometric scanning of the autoradiograms. The readings were taken from a linear scale. Deletion of the four 3′-most nucleotides (MT 1/14) or the two 5′-most nucleotides (MT 19) had only a minor effect on the amount of subgenomic DI RNA synthesized, whereas deletion of both of these regions (MT 18) significantly affected transcription levels. These data were consistent with previous studies (18) and further confirmed the observation that the flanking sequences outside the intergenic region do not play a role in subgenomic DI RNA transcription.

To examine the minimum nucleotide sequence requirement for subgenomic DI RNA transcription and the effects of nucleotide mutations within the intergenic sequence on subgenomic DI RNA transcription, seven additional MHV DI cDNA clones were created. MT 21, MT 22, MT 23, and MT 25 all had the complete UCUAAAC consensus sequence but had different upstream and downstream nucleotides (Fig. 3A). MT 26, MT 27, and MT28 lacked a complete consensus sequence and contained mutated nucleotides within the consensus sequence. Northern blot analysis of these clones demonstrated that only a minute amount of subgenomic DI RNAs was synthesized in MT 21-, MT 22-, MT 23-, and MT 25-replicating cells, whereas subgenomic DI RNA synthesis was not detected at all in MT 26- and MT 28-replicating cells (Fig. 3B). These data indicated that the presence of UCU AAAC is necessary for subgenomic DI RNA synthesis. Interestingly, a small amount of subgenomic DI RNA was also observed in the cells which were infected with the virus from MT 27 DI RNA-transfected cells, suggesting that GCUAAAC may also be recognized as a functional sequence. The Northern blot data were further examined by PCR. RT-PCR was performed with use of the intracellular RNA species from passage 1 virus-infected cells and oligonucleotides 1111 and 78 as primers. As shown in Fig. 4, the PCR product of the predicted size was not detected in the sample obtained from the MT 26- and MT 28-derived passage 1 virus-infected cells, whereas the PCR product of the predicted size was demonstrated in the sample obtained from MT 25 and MT 27-infected cells, confirming the Northern blot analysis.

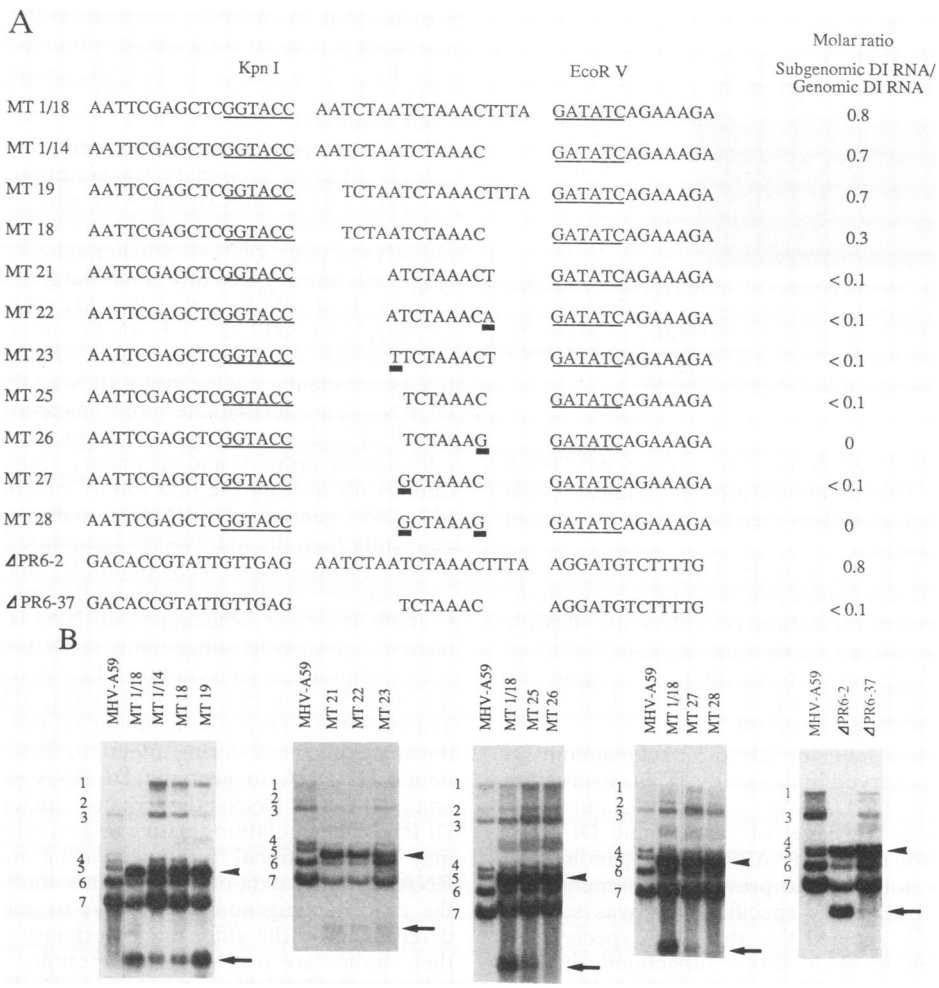

FIG. 3. Analysis of DI RNAs which lack intergenic region flanking sequences. (A) Sequences at the inserted intergenic sequences of MHV DI cDNAs. *Kpn*I and *Eco*RV sites are underlined. Mutated nucleotides are shown by underbars. (B) Northern blot analysis of mutant DI RNAs listed in panel A. Passage 1 virus samples were used as inocula. Intracellular RNAs were extracted at 7 h postinfection, separated by formaldehyde–1% agarose 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 to 7 indicate MHV-A59-specific mRNA species. Arrowheads and arrows point to genomic DI RNA and subgenomic DI RNAs, respectively.

It was observed that PR6 mutant 3 DI RNA, which contains the intergenic consensus sequence UCUAAAC but lacks other sequence within the intergenic sequence, does not produce a detectable level of subgenomic DI RNA (18). Since synthesis of the subgenomic DI RNA from MT 25 was detected in the present study, whether the DI RNA with the intergenic consensus sequence UCUAAAC and intergenic region flanking sequences, but without other nucleotides within the intergenic sequence, synthesized subgenomic DI RNA was reexamined. The PR6 mutant 3-derived ΔPR6-37 (8) has the structure of ΔPR6-2, except that ΔPR6-37 contains only the consensus sequence UCUAAAC within the intergenic region (Fig. 3A). Unexpectedly, synthesis of trace amounts of subgenomic DI RNA was detected in the cells which were infected with passage 1 virus samples derived from ΔPR6-37-transfected cells (Fig. 3B). Also, a similar level of subgenomic DI RNA transcription was observed in cells infected with passage 1 virus from PR6 mutant 3-transfected cells (data not shown). These studies demonstrated that the UCUAAAC consensus sequence was sufficient for subgenomic DI RNA transcription.

DISCUSSION

This study demonstrated that for the 18-nucleotide-long, gene 6-7 intergenic sequence in DI RNA, the presence or absence of upstream and downstream flanking sequences, of the sizes studied, did not significantly alter the ratio of subgenomic DI RNA to genomic DI RNA. Furthermore, it was found that the UCUAAAC consensus sequence was sufficient for a low level of subgenomic DI RNA transcription. The data suggested that the sequences flanking the intergenic sequence preceding gene 7 do not significantly regulate MHV mRNA 7 transcription. The possibilities that another genomic region(s) regulates subgenomic mRNA 7 synthesis (see below) and that synthesis of other MHV subgenomic mRNAs is affected by their intergenic region flanking sequences cannot be ruled out. The absence of enhancer sequences for MHV subgenomic RNA transcription near the genes 6-7 intergenic region contrasted with the data from other subgenomic RNA-synthesizing positive-stranded RNA viruses of the alphavirus superfamily (1). These viruses have relatively long sequences surrounding a

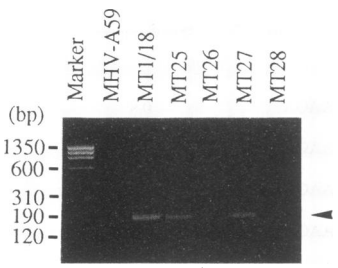

FIG. 4. Agarose gel electrophoresis of PCR products amplified from DI RNA-replicating cells. Oligonucleotides 1111 and 78 were used for RT-PCR. The PCR products of expected size are shown by an arrowhead. The size marker is *Hae*III-digested ϕX174 DNA (Promega). DNA was stained with ethidium bromide.

core promoter sequence which increases subgenomic RNA transcription (5, 17). This difference probably indicates that coronavirus transcription differs significantly from that of the alphavirus superfamily.

The data obtained from the analysis of MT 27, MT 21, MT 22, MT 23, and MT 24 were consistent with the observations that MHV transcription regulation is sufficiently flexible enough to recognize altered consensus sequences (9) and that sequence homology between the intergenic region and the 5′-end genomic leader sequence is not the sole determinant of subgenomic RNA quantity (9).

Unexpectedly, a low level of ΔPR6-37 subgenomic DI RNA synthesis was observed in this study. This observation demonstrated that the UCUAAAC consensus sequence is indeed sufficient for a low level of subgenomic DI RNA transcription. It is not clear why ΔPR6-37 subgenomic DI RNA synthesis was not detected previously. Sometimes a small amount of a 1-kb MHV-specific RNA was seen in MHV-infected cells. It seemed that this RNA species was not a DI RNA but more likely was a subgenomic mRNA species not described before. The emergence of this RNA species was sporadic (unpublished data). It is possible that minor variations in experimental conditions are crucial for the synthesis of subgenomic RNA species not observed consistently.

Transcription enhancer elements were not detected; therefore, it is still not clear why the ratio of subgenomic DI RNA to genomic DI RNA was so drastically different from the ratio of mRNA 7 to mRNA 1. One possibility is that the amount of helper virus-derived MHV transcription function in DI RNA-replicating cells is too low for efficient subgenomic DI RNA transcription. MHV DI RNA replication strongly inhibits helper MHV RNA replication and transcription (24). Perhaps this means that significantly decreased amounts of MHV-specific transcription factors are present in DI RNA-replicating cells such that MHV transcriptional function are not sufficient for recognition of the template DI RNA molecules and subsequent subgenomic DI RNA transcription. Another possibility is that a host derived-factor(s) limits the efficiency of MHV RNA transcription. If MHV RNA transcription takes place at specific sites within the cells or requires a host-derived factor(s) or both, then the maximum number of RNA molecules involved in MHV RNA transcription may be saturated after the accumulation of large amounts of template RNA molecules. Although genomic DI RNA accumulates more efficiently than MHV genomic RNA (8), only a small fraction of the DI RNA would be used as a transcription template because of the limited availability of host functions. Alternatively, it is possible that an enhancer sequence(s) for subgenomic RNA synthesis is present somewhere within genes 1 to 4 of MHV genomic RNA. The lack of such a sequence(s) in the DI RNA might result in a lower efficiency of subgenomic DI RNA synthesis.

It was proposed that coronavirus subgenomic RNA is amplified during secondary transcription (31). This model, however, does not explain well why the molar ratio of coronavirus subgenomic mRNA is essentially constant during MHV replication (16). Smaller subgenomic RNA may be amplified more efficiently than larger subgenomic RNA species. It should be noted that MT 1440/453, MT283/453, ΔPR6-2, and MT1/453 DI RNAs are of different sizes, whereas the sizes and structures of their subgenomic DI RNAs are identical. One study showed that a smaller MHV DI RNA does accumulate more efficiently than a larger DI RNA (8) and that MHV transcription activities, including initiation of primary and secondary transcription, are continuous for at least the first 6 h of infection (8). Therefore, MT 1/453 genomic DI RNA probably accumulated more efficiently than the MT 1440/453 genomic DI RNA during DI RNA replication, and subgenomic DI RNA was most likely continuously synthesized from replicating genomic DI RNA. If amplification of subgenomic mRNAs is the major mechanism of coronavirus subgenomic mRNA accumulation, then why is the ratio of subgenomic DI RNA to genomic DI RNA the same for constructs MT 1440/453, MT283/453, ΔPR6-2, and MT1/453? If these subgenomic DI RNAs are amplified at the same efficiency during infection, then the ratio of subgenomic DI RNA to genomic DI RNA should be different among these DI RNAs; the reduced efficiency of subgenomic DI RNA accumulation relative to genomic DI RNA quantity may be proportional to the decreasing order of genomic DI RNA size. The data obtained in this study may suggest that the ratio of subgenomic DI RNA to genomic DI RNA is determined by the efficiency of primary transcription. Further studies are necessary to determine why the ratios of subgenomic DI RNA to genomic DI RNA remain stable among the mutant DI RNA constructs.

ACKNOWLEDGMENTS

We thank Michael Lai and Chien-Kou Shieh for Am 20. We thank Kyongmin Hwang for the JW 2 clone and Jennifer Fosmire for helpful suggestions on the manuscript.

This work was supported by Public Health Service grant AI29984 from the National Institutes of Health.

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