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Coronavirus Transcription Mediated by Sequences Flanking the Transcription Consensus Sequence

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In our studies of murine coronavirus transcription, we continue to use defective interfering (DI) RNAs of mouse hepatitis virus (MHV) in which we insert a transcription consensus sequence in order to mimic subgenomic RNA synthesis from the nondefective genome. Using our subgenomic DI system, we have studied the effects of sequences flanking the MHV transcription consensus sequence on subgenomic RNA transcription. We obtained the following results. (i) Insertion of a 12-nucleotide-long sequence including the UCUAAAC transcription consensus sequence at different locations of the DI RNA resulted in different efficiencies of subgenomic DI RNA synthesis. (ii) Differences in the amount of subgenomic DI RNA were defined by the sequences that flanked the 12-nucleotide-long sequence and were not affected by the location of the 12-nucleotide-long sequence on the DI RNA. (iii) Naturally occurring flanking sequences of intergenic sequences at gene 6–7, but not at genes 1–2 and 2–3, contained a transcription suppressive element(s). (iv) Each of three naturally occurring flanking sequences of an MHV genomic cryptic transcription consensus sequence from MHV gene 1 also contained a transcription suppressive element(s). These data showed that sequences flanking the transcription consensus sequence affected MHV transcription. © 1996 Academic Press, Inc.

INTRODUCTION

Mouse hepatitis virus (MHV), a coronavirus, is an enveloped virus with a single-stranded, positive-sense RNA genome of approximately 31 kb (Lee *et al.*, 1991; Pachuk *et al.*, 1989). MHV-infected cells synthesize genomic-length virus-specific mRNA and six or seven species of virus-specific subgenomic mRNAs. The viral mRNAs have a 3'-coterminal nested-set structure (Lai *et al.*, 1981; Leibowitz *et al.*, 1981) and are numbered 1 to 7, in decreasing order of size (Lai *et al.*, 1981; Leibowitz *et al.*, 1981). None of the mRNAs are packaged into MHV virions, except for mRNA 1, which is efficiently packaged due to the presence of a packaging signal (Fosmire *et al.*, 1992). The 5' ends of the MHV genomic RNA and the subgenomic mRNAs start with a leader sequence that is approximately 72 to 77 nucleotides long (Lai *et al.*, 1983, 1984; Spaan *et al.*, 1983). The leader sequence is encoded only once in the genomic RNA at the 5' end. The MHV-specific genes, which are downstream from the leader, are separated from one another by an intergenic region. Each intergenic region, located upstream of a gene essential for MHV replication, includes the unique consensus sequence of UCUAAAC, or a very similar sequence (Lai *et al.*, 1984; Spaan *et al.*, 1983). On the subgenomic mRNAs, the leader sequence is fused to the

intergenic consensus sequence, which marks the start of the gene (Joo and Makino, 1992; Lai *et al.*, 1984; Makino *et al.*, 1988b; Shieh *et al.*, 1987; Spaan *et al.*, 1983).

MHV subgenomic RNAs are detected in MHV-infected cells but not in MHV virion (Lai and Stohman, 1978). Therefore, subgenomic-sized RNAs must be synthesized from a genomic-sized RNA. Synthesis of subgenomic mRNAs involves a discontinuous transcription step; during subgenomic-sized RNA synthesis independently transcribed leader RNA species possessing a trans-acting property fuse with the body sequences of subgenomic-sized RNA (Jeong and Makino, 1994; Zhang *et al.*, 1994). There are at least two stages in coronavirus subgenomic RNA synthesis: we call the first stage primary transcription, during which subgenomic-sized RNA is synthesized from a genomic-sized template RNA; the other stage is called secondary transcription, during which subgenomic-sized RNA serves as template (Jeong and Makino, 1992).

When an intergenic region from MHV is inserted into a location in an MHV defective interfering (DI) RNA, a novel subgenomic DI RNA is transcribed in helper virus-infected cells (Makino *et al.*, 1991). We used this subgenomic DI RNA system to study how MHV transcription is flexible enough to recognize a mutated transcription consensus sequence. We constructed a series of MHV DI RNAs that contain one UCUAAAC consensus sequence with a single-nucleotide mutation in the middle of the 0.3-kb-long intergenic region between genes 6 and 7 (Joo and Makino, 1992). Analysis of these mutant DI

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RNAs showed that the MHV transcription mechanism is flexible enough to recognize mutated transcription consensus sequences; subgenomic DI RNAs are synthesized from most of the mutated consensus sequences (Joo and Makino, 1992). In another study we showed that sequences flanking the same intergenic region between genes 6 and 7 do not affect the efficiency of subgenomic DI RNA transcription (Makino and Joo, 1993). These two studies, however, do not explain why most of the genomic cryptic consensus sequences that are very similar to the UCUAAC consensus sequence are not recognized for MHV transcription. MHV genomic RNA contains 19 regions, in which only one nucleotide differs from the UCUAAC sequence (Joo and Makino, 1992), and these regions are not utilized for transcription.

One of the possible reasons why transcription does not occur in these 19 MHV genomic regions is that the flanking sequences of these regions suppress transcription. In both of the previous studies we used DI RNAs which contain the intergenic region between genes 6 and 7; we used that intergenic region because mRNA 7, which is synthesized from this intergenic region, is the most abundant MHV mRNA species and we expected that a large amount of subgenomic DI RNA would be synthesized from this inserted intergenic region. However, characterization of only this intergenic region may overlook the possibility that the sequences flanking these 19 regions may suppress transcription from these regions.

Deletion analysis of those MHV DI RNAs that contain the intergenic sequence from gene 6–7 with its naturally occurring flanking sequences showed that reducing the number of base pairs between the genomic leader sequence and the intergenic region decreases the transcription efficiency (Makino *et al.*, 1991). However, when van der Most *et al.* (1994) used another MHV DI RNA, those authors reported that the extent of base pairing between the leader RNA and the intergenic sequence does not control subgenomic RNA abundance; they used DI RNAs which lacked the naturally occurring sequences that flank that intergenic sequence. If the flanking sequences of the transcription consensus sequence affect transcription, then the different results from these two studies may be explained by the differences in the nature of sequences flanking the inserted intergenic sequence.

We have studied the possible influence of the flanking sequences on transcription initiated at a transcription consensus sequences by using a subgenomic DI system. Our data indicated that indeed some flanking sequences affected transcription.

MATERIALS AND METHODS

Viruses and cells

The plaque-cloned A59 strain of MHV (MHV-A59) (Lai *et al.*, 1981) was used as a helper virus. Mouse DBT cells (Hirano *et al.*, 1974) were used for growth of viruses.

DNA construction

MHV DIssE-specific cDNA clone DE5-w3 (Makino and Lai, 1989) was used as a parental clone for DNA construction. Conventional methods of DNA manipulation (Sambrook *et al.*, 1989) were used. By using PCR-based site-directed mutagenesis, a 12-nucleotide-long sequence, TCTAATCTAAAC, was inserted into DI cDNA (Joo and Makino, 1992). For PCR the DNA was incubated with two oligonucleotides in PCR buffer (0.05 M KCl, 0.01 M Tris-HCl (pH 8.3), 0.0025 M MgCl₂, 0.01% gelatin, 0.17 mM each of dNTPs, and 5 U of Taq polymerase (Promega)) at 93° for 30 sec, 37° for 45 sec, and 72° for 100 sec for total of 30 cycles. A procedure based on recombinant PCR was also employed for construction of plasmid DI-D20 and DI-TA7 (Higuchi, 1990). For the construction of FDI-1/2wt, FDI-2/3wt, FDI-6/7wt, FDI-M1, FDI-M2, and FDI-M3, MHV-JHM-specific cDNA was initially synthesized by incubating MHV-JHM genomic RNA with specific primers (Makino *et al.*, 1988a), the desired MHV-JHM-specific RT-PCR products were obtained after incubating MHV-JHM cDNA with two specific oligonucleotides, as described previously (Makino and Joo, 1993). For construction of FDI-1/2M, FDI-2/3M, and FDI-6/7M the 12-nucleotide-long sequence, TCTAATCTAAAC, was inserted into the RT-PCR products using the recombinant PCR procedure. The recombinant PCR procedure was also used to insert a TCTTAAC sequence into FDI-M5. The resulting RT-PCR product was inserted into the *Afl*III–*Sac*II site of DE5-w3. For all of the constructs used in this study we sequenced the inserts that were derived from PCR products to confirm the presence of specific mutations and the absence of extraneous mutations.

RNA transcription and transfection

Plasmid DNAs were linearized by *Xba*I digestion and transcribed with T7 RNA polymerase as previously described (Makino and Lai, 1989). The lipofection procedure (Makino *et al.*, 1991) was used for RNA transfection into DBT cells.

Preparation of virus-specific intracellular RNA and Northern (RNA) blotting

Virus-specific RNAs in virus-infected cells were extracted as previously described (Makino *et al.*, 1984). 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 as described previously (Jeong and Makino, 1992). In some experiments poly(A) containing RNAs that were selected by oligo (dT)-cellulose column chromatography were used (Makino *et al.*, 1984). The nylon filter was soaked in a prehybridization buffer, and Northern blot hybridization was performed (Jeong and Makino, 1992). The ³²P-labeled probes were prepared by

a random-priming procedure (Sambrook *et al.*, 1989). For the densitometric analysis, autoradiograms were scanned using a scanner (RELI 4816 scanner, Relysis) and the intensity of each band was quantitated using Scan Analysis program (Biosoft, Cambridge, UK).

PCR and Direct sequencing of the PCR products

Primer extension products were purified from the gel and amplified by PCR under the same conditions described above. The gel-purified RT-PCR products were separated by agarose gel electrophoresis. Direct PCR sequencing was performed according to the procedure established by Winship (1989).

RESULTS

Insertion of the transcription consensus sequence at different locations in an MHV DI RNA

We examined whether sequences outside of the consensus sequence could affect subgenomic RNA transcription efficiency by using the subgenomic DI RNA system. We initially constructed eight different MHV DI cDNA clones, each with a 12-nucleotide-long TCTAATCTAAAC insert (12-nt sequence) placed at a different site; that inserted 12-nt sequence is perfectly complementary to the 3' region of the genomic leader sequence and includes the transcription consensus sequence UCUAAAC. This 12-nt sequence, therefore, was located differently and flanked differently in each of the DI RNA constructs; its flanking sequences depended upon its location. A 2.2-kb-long MHV DIssE cDNA clone, DE5-w3, was used as a parental clone (Fig. 1A) (Makino and Lai, 1989). DIssE consists of three noncontiguous regions of the helper virus genome (Makino *et al.*, 1988a) and includes cis-acting replication signals, which are essential for MHV-JHM DI RNA replication; these regions were the 5'-most 0.47 kb, the 3'-most 0.46 kb, and about 60 nucleotides near the 5' end of domain II (Kim *et al.*, 1993; Kim and Makino, 1995b; Lin and Lai, 1993) (Fig. 1A). We used PCR-based site-directed mutagenesis to insert the 12-nt sequence into DE5-w3. The locations of the 12-nt sequences in these eight clones, DI-AflIII, DI-StuI, DI-EagI, DI-SphI, DI-SpeI, DI-D20, DI-NruI, and DI-TA 7, are shown in Fig. 1B.

We examined replication and transcription of these DI RNAs in DI RNA-transfected, MHV-infected cells. DI RNAs were synthesized *in vitro* and transfected by lipofection into DBT cell monolayers that were infected with MHV-A59 helper virus 1 hr prior to transfection. At 7 hr postinfection, intracellular RNAs were extracted, separated by formaldehyde-agarose gel electrophoresis, and analyzed by Northern blot. For the analysis of DI-AflIII, DI-StuI, and DI-EagI, we electrophoresed poly(A)-containing intracellular RNAs and probed Northern blots with the *EagI-SphI* DNA fragment of DE5-w3 (Fig. 1A) that was

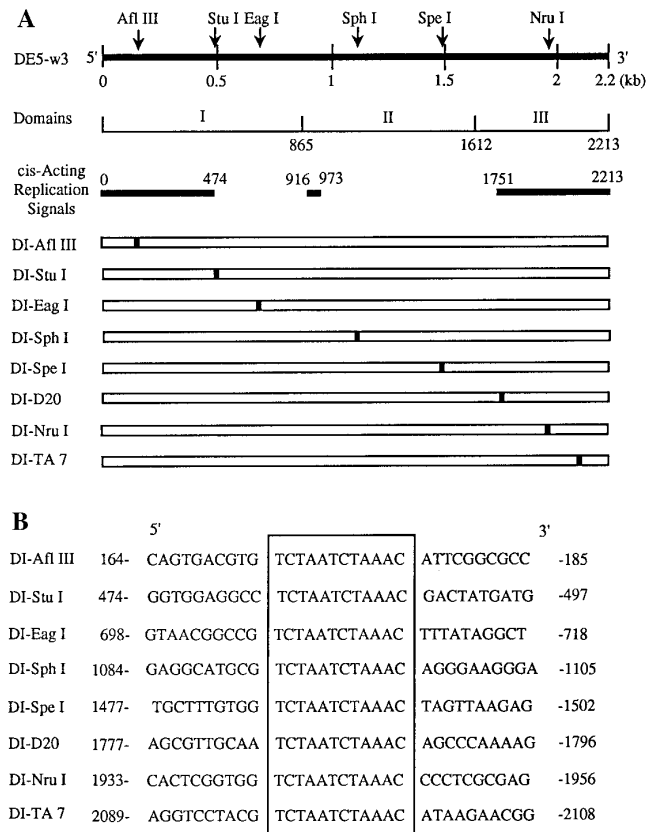


FIG. 1. Diagram of the structure of MHV DI cDNAs. (A) Schematic diagram of the structure of DE5-w3 and DE5-w3-derived insertion mutants. Restriction enzyme sites used for the construction of the insertion mutants are shown at the top of the diagram. The three domains (Domain I through III) are indicated and the cis-acting MHV DI RNA replication signals (Kim *et al.*, 1993; Kim and Makino, 1995b) are shown. Solid boxes in the insertion mutants represent the positions of the 12-nucleotide sequence insertion. (B) Sequences adjacent to the inserted 12-nucleotide sequence are shown for each insertion mutant. Nucleotide numbering begins from the 5'-end of DE5-w3, and the inserted 12-nucleotide sequences are boxed.

labeled by random-priming with ^{32}P . This probe detects only helper virus genomic RNA, genomic DI RNAs, and subgenomic DI RNAs (Fig. 2A). For the detection of the remaining DI mutants, we used the *EagI-SphI* fragment of DE5-w3 as a probe (Fig. 2C), and a probe corresponding to a region 18 to 262 nucleotides from the 3'-end of DE5-w3 (Figs. 2B and 2D); the former probe detects only helper virus mRNA 1 and genomic DI RNAs and the latter binds to all MHV RNA species. To directly compare the efficiency of subgenomic DI RNA transcription, we used the same membrane in the experiments documented in Figs. 2B and 2C. All eight DI RNAs replicated efficiently and synthesized a subgenomic DI RNA of expected size; however, the amounts of the subgenomic DI RNAs differed (Fig. 2). Subgenomic DI RNA was most efficiently transcribed from DI-SpeI DI RNA. DI-SphI and DI-D20 also supported subgenomic DI RNA transcription. DI-NruI supported a low level of subgenomic DI RNA tran-

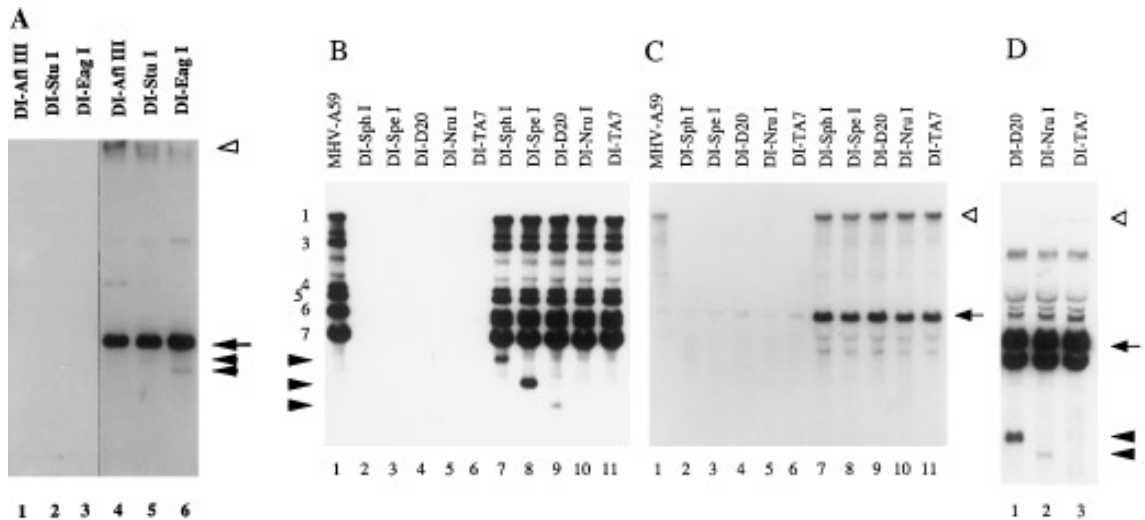


FIG. 2. Northern blot analysis of DE5-w3-derived mutant subgenomic DI RNAs. Equal amounts of each *in vitro*-synthesized DI RNA were transfected into MHV-A59-infected cells (A, lanes 4–6; B, lanes 7–11; C, lanes 7–11; D, lanes 1–3) or mock-infected (A, lanes 1–3; B, lanes 2–6; C, lanes 2–6) DBT cells at 1 hr postinfection. Intracellular RNA was extracted at 7 hr postinfection, separated by formaldehyde-agarose gel electrophoresis, and transferred to a nylon membrane. Lanes 1 of B and C represent RNA from MHV-infected cells. The probes were prepared by random-primed ^{32}P labeling of the *EagI*–*SphI* (A and C) and 3'-end 0.25-kb (B and D) fragments of DE5-w3 DNA. Analysis of poly(A)-containing RNAs, A: cytoplasmic RNA, B, C, D. B and C show experimental results from the same membrane. Arrowhead, arrow, and open triangles point to subgenomic DI RNAs, genomic DI RNAs, and helper virus genomic RNAs, respectively. Numbers 1, 3, 4, 5, 6, and 7 represent major MHV-A59-specific mRNA species.

scription, which was not immediately apparent in one experiment, as shown in Fig. 2B, in which it could only be detected after prolonged exposure of the gel (data not shown); in another experiment, DI-*NruI* subgenomic DI RNA synthesis was observed more readily, because the autoradiogram had a lower background (Fig. 2D). A lower level of subgenomic DI RNA synthesis was also observed in DI-*Stul* and DI-*Eagl*. Although synthesis of DI-*AflIII* and DI-*TA7* subgenomic DI RNAs was not evident by Northern blot analysis, synthesis of both subgenomic DI RNAs was detected by RT-PCR analysis (Jeong and Makino, 1994) (data not shown). Direct sequencing of genomic DI RNA-specific RT-PCR products demonstrated that the inserted 12-nt sequence and its flanking regions was maintained for all the mutants in DI RNA-replicating cells (data not shown).

Flanking sequences of the 12-nt sequence affected transcription

To test whether the flanking sequences of the inserted 12-nt sequence affected the efficiency of transcription, we constructed another set of DI cDNAs. From DI cDNAs, DI-*Stul*, DI-*Eagl*, DI-*SphI*, DI-*SpeI*, DI-D20, and DI-*NruI*, we removed a 0.4-kb-long PCR fragment, which carried the 12-nt sequence and 0.2 kb of its upstream and downstream flanking sequences, and inserted this into the *AflIII*–*SacII* site of DE5-w3 to produce FDI-*Stul*, FDI-*Eagl*, FDI-*SphI*, FDI-*SpeI*, FDI-D20, and FDI-*NruI*, respectively (Fig. 3). These six cDNAs were the same size, and each carried the same 12-nt sequence in the middle of a same-length insertion; only the regions flanking the 12-nt se-

quence insertion differed. If the sequences flanking the 12-nt sequence are important for subgenomic DI RNA transcriptional regulation, then the efficiency of subgenomic DI RNA transcription from the newly constructed DI RNAs should be similar to that of their parental DI RNAs.

We examined synthesis of subgenomic DI RNA in DI RNA-transfected, MHV-A59-infected cells by Northern blot analysis in which we applied only poly(A)-containing RNAs onto the gels. We used probe 1 (see Fig. 3) to estimate the ratio of genomic to subgenomic DI RNA for most of the DI RNAs; the exception was that we used probe 2 (see Fig. 3) for the analysis of FDI-D20. Probe 1 was not suitable for analysis of FDI-D20, because while this probe hybridizes with genomic FDI-D20 RNA at two different sites (one is downstream of the *AflIII* site and the other is downstream of the *SacII* site), it hybridizes with the subgenomic DI RNA at just a single site. All the tested DI RNAs replicated efficiently in the DI RNA-transfected, MHV-A59-infected cells (Fig. 4). A more slowly migrating band appeared in FDI-*SpeI* DI RNA-replicating cells, in FDI-D20 DI RNA-replicating cells and in FDI-*NruI* DI RNA-replicating cells (Fig. 4, lanes 5–7); this band probably represented a DI RNA that was newly generated in DI RNA-transfected cells. All the DI RNA-replicating cells synthesized subgenomic DI RNA with different transcription efficiencies. The difference in the radioactivity ratios of subgenomic DI RNA to genomic DI RNA for these five DI RNAs was roughly comparable to those of the parental DI RNAs (Figs. 2 and 4). The 0.2-kb-long upstream and downstream sequences flanking the 12-nt sequence clearly affected subgenomic DI RNA transcription efficiency.

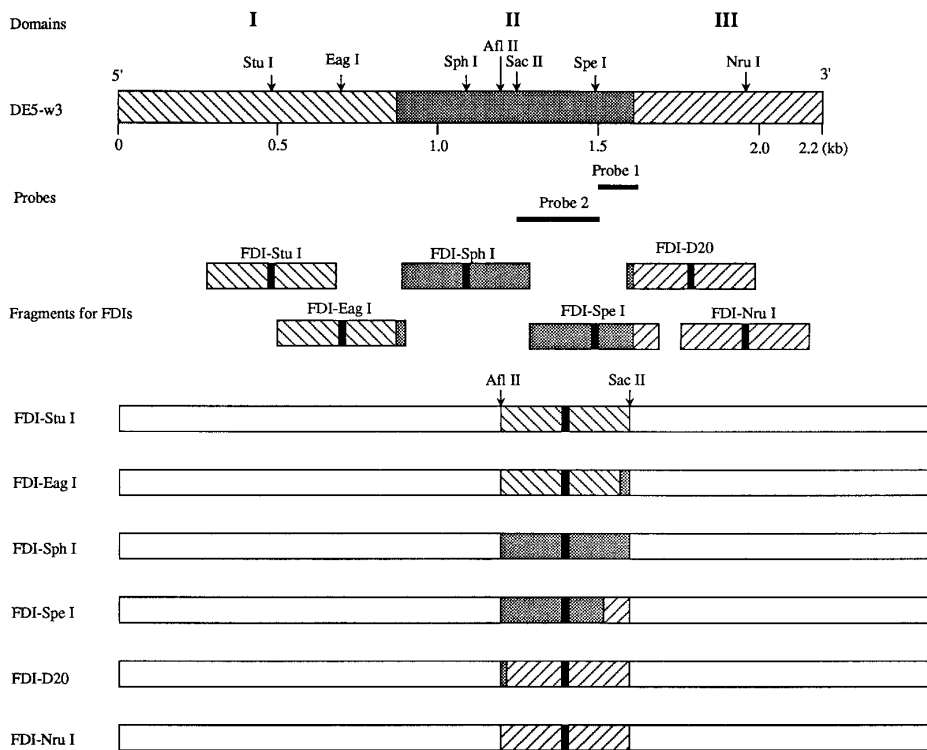


FIG. 3. Schematic diagram of the structure of DE5-w3 and DE5-w3-derived insertion mutants with flanking sequences. Fragments for FDI contained a 0.4-kb-long PCR product consisting of the 12-nucleotide sequence (solid box) and 0.2 kb from an upstream and a downstream flanking sequence; the flanking sequences were derived from DI-*Stul*, DI-*Eagl*, DI-*SphI*, DI-*SpeI*, DI-D20, and DI-*Nrul* genomic fragments. The 0.4-kb PCR fragment of DI-*Stul*, DI-*Eagl*, DI-*SphI*, DI-*SpeI*, DI-D20, and DI-*Nrul* was inserted into the *AflII*-*SacII* site of the DE5-w3 to produce FDI-*Stul*, FDI-*Eagl*, FDI-*SphI*, FDI-*SpeI*, FDI-D20, and FDI-*Nrul*, respectively. Solid boxes represent the 12-nucleotide sequence. Locations of probe 1 and probe 2 used for Northern blot analysis (see Fig. 4) are also shown.

Effect of the 12-nt sequence location on transcription

We next examined whether the location of the 12-nt sequence could affect transcription by constructing a new series of DI cDNAs with the 12-nt sequence positioned at different sites in DE5-w3; each construct was similar to the others in that they all contained the 0.4-kb inserted region that carried the 12-nt sequence and its upstream and downstream 0.2-kb sequences; each construct differed only in the location of the insertion within the DI genome. We chose the 0.4-kb fragment used for construction of FDI-*SpeI*, because FDI-*SpeI* showed the most efficient subgenomic DI RNA transcription (Fig. 4). We inserted this 0.4-kb fragment at the *AflIII*, *Stul*, *Eagl*, *SphI*, *AflII*, *SacII*, and *Nrul* sites of DE5-w3 to produce FDI-*AflIII/Sp*, FDI-*Stul/Sp*, FDI-*Eagl/Sp*, FDI-*SphI/Sp*, FDI-*AflII/Sp*, FDI-*SacII/Sp* and FDI-*Nrul/Sp*, respectively (Fig. 5A). We expected that this set of DI constructs would overcome the transcriptional suppressive effect that some sequences flanking the 12-nt sequence exerted and thereby reveal whether location of the 12-nt sequence was affecting subgenomic DI RNA synthesis.

Synthesis of subgenomic DI RNA in DI RNA-transfected, MHV-A59-infected cells was examined by Northern blot analysis. We applied poly(A)-containing intracellular RNA species on the gels and used 32 P-labeled

probe 1 (Fig. 5A). FDI-*Stul/Sp*, FDI-*Eagl/Sp*, FDI-*SphI/Sp*, FDI-*AflII/Sp*, and FDI-*SacII/Sp* replicated and transcribed (Fig. 5B). The molar ratio of the genomic to subgenomic

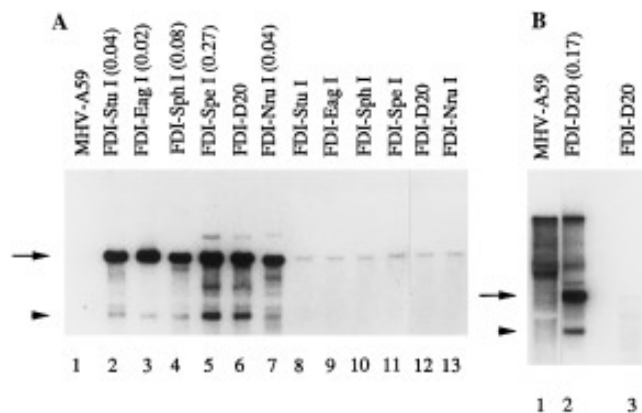


FIG. 4. Northern blot analysis of FDI RNAs. Intracellular RNAs were extracted from DI RNA-transfected, MHV-infected cells (A, lanes 2–7; B, lane 2) or DI RNA-transfected, mock-infected cells (A, lanes 8–13; B, lane 3) and poly(A)-containing RNAs were analyzed by Northern blot analysis. Lanes 1 of both panels represent RNA from MHV-infected cells. Probe 1 and probe 2 (see Fig. 3) were used for detection of DI RNAs in A and B, respectively. Arrowhead and arrow point to subgenomic DI RNAs and genomic DI RNAs, respectively. The molar ratios of genomic DI RNA to subgenomic DI RNA are shown in parentheses.

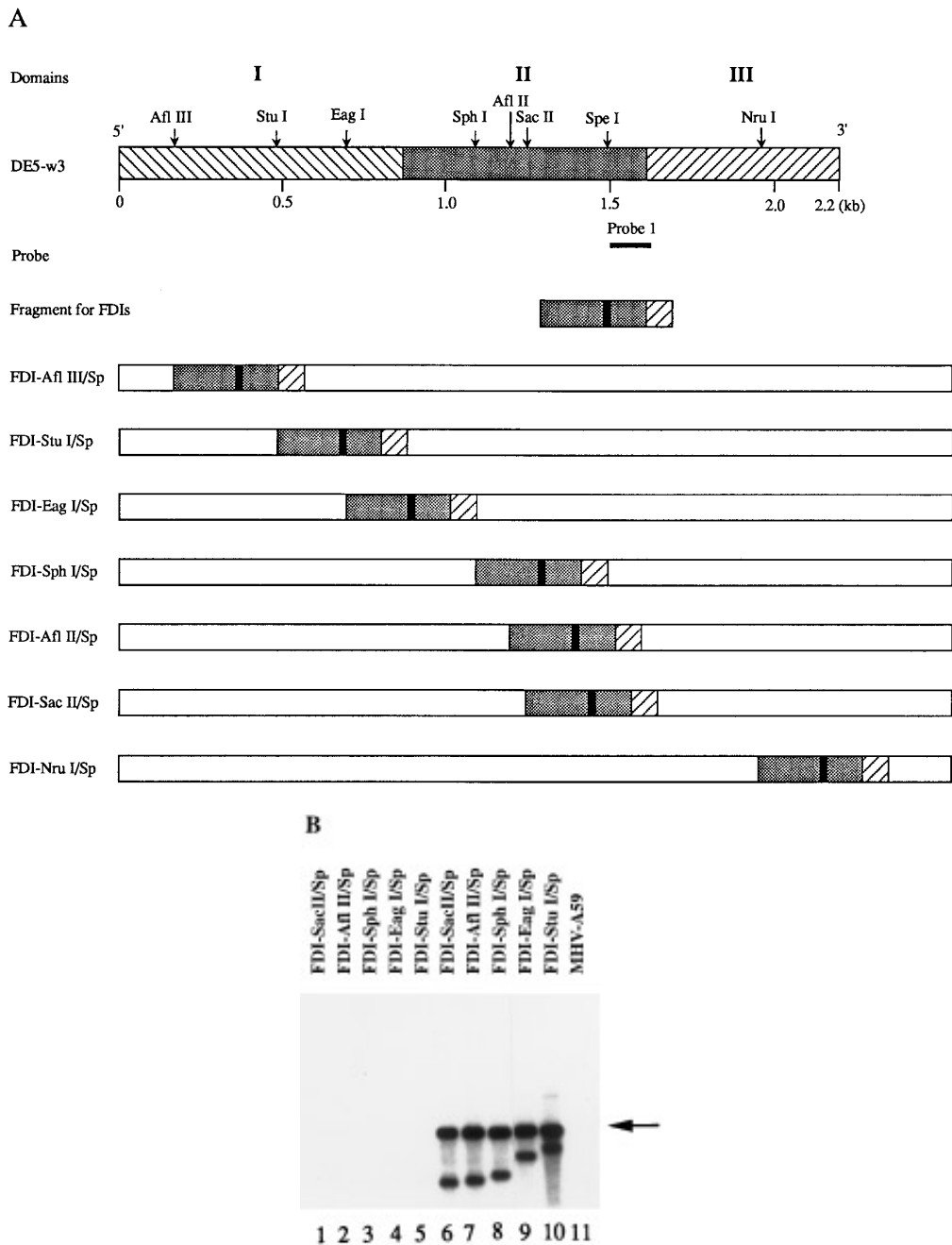


FIG. 5. Effect of location of the 0.4-kb region on subgenomic DI RNA transcription. (A) Schematic diagram of the structure of DE5-w3 and DE5-w3-derived insertion mutants with flanking sequences. Fragment for FDIs contained a 0.4-kb-long PCR product consisting of the 12-nucleotide sequence (solid box) and 0.2 kb from an upstream and a downstream flanking sequence of DI-*SpeI* genomic fragments. This 0.4-kb PCR fragment was inserted into the *Afl*III site, *Stu*I site, *Eag*I site, *Sph*I site, *Afl*II site, *Sac*II site, and *Nru*I site of the DE5-w3 to produce FDI-*Afl*III/Sp, FDI-*Stu*I/Sp, FDI-*Eag*I/Sp, FDI-*Sph*I/Sp, FDI-*Afl*II/Sp, FDI-*Sac*II/Sp, and FDI-*Nru*I/Sp, respectively. Solid boxes represent the 12-nucleotide sequence. Location of probe 1 for Northern blot analysis is also shown. (B) Northern blot analysis of FDI RNAs. Intracellular RNAs were extracted from DI RNA-transfected, MHV-infected cells (lanes 6–10) or DI RNA-transfected, mock-infected cells (lanes 1–5), and poly(A)-containing RNAs were analyzed by Northern blot analysis. Lane 11 represents RNA from MHV-infected cells. 32 P-labeled probe 1 (see A) was used as a probe. Arrow represents genomic DI RNAs.

DI RNA from these replication-competent DI RNAs was essentially the same (Fig. 5B); the ratio was approximately 0.3, a value that was very close to that of FDI-*SpeI*. We did not observe replication of FDI-*Afl*III/Sp and FDI-*Nru*I/Sp (data not shown). The 0.4-kb region was

inserted within the cis-acting replication signals in these DI RNAs (Kim *et al.*, 1993) (Fig. 1). Probably the insertion of the 0.4 kb disrupted the structure of the cis-acting replication signals in regions that were essential for DI RNA replication, resulting in failure of the DI RNA to

replicate. These data indicated that the location of 12-nt sequence on DE5-w3 was not crucial for the regulation of subgenomic DI RNA transcription.

Effect of the naturally occurring sequences that flank the MHV intergenic regions on transcription

The data presented above demonstrated that some sequences flanking the 12-nt sequence could suppress subgenomic DI RNA transcriptional efficiency. This data contrasted with our previous observation that the efficiency of subgenomic DI RNA transcription is not regulated by sequences flanking the genes 6–7 intergenic sequence (Makino and Joo, 1993). Here we present that not all sequences flanking the 12-nt sequence inhibited the transcription; some sequences flanking the 12-nt sequence did not suppress transcription (see Fig. 4). We interpreted these data as indicating that nonnaturally occurring sequences flanking the 12-nt sequence could suppress subgenomic DI RNA transcription, whereas naturally occurring sequences flanking the genes 6–7 intergenic sequence do not suppress transcription.

We examined naturally occurring flanking sequences from other MHV intergenic regions. For this analysis we chose sequences surrounding the genes 1–2 intergenic sequence and the genes 2–3 intergenic sequence. The amounts of mRNA 2 and mRNA 3, synthesized from the intergenic regions between genes 1–2 and genes 2–3, respectively, are about 50 and 30 times lower than the amount of mRNA 7 in MHV-infected cells (Leibowitz *et al.*, 1981). Currently we do not know why the amount of mRNA 2 and mRNA 3 is significantly lower than mRNA 7. We constructed six different DI cDNAs, all of which had an insertion of a 0.4-kb fragment at the *Afl*II–*Sac*II site of DE5-w3 (see Figs. 3 and 6). The inserted 0.4-kb fragment of FDI-1/2wt, FDI-2/3wt, and FDI-6/7wt, was a RT-PCR fragment of the MHV-JHM sequence at genes between 1–2, 2–3, and 6–7, respectively (Fig. 6). The 0.4-kb fragment consisted of the intergenic sequence and its natural occurring 0.2-kb flanking sequences. FDI-1/2M, FDI-2/3M, and FDI-6/7M had structures that, respectively, were very similar to FDI-1/2wt, FDI-2/3wt, and FDI-6/7wt, except that these DI cDNAs contained the 12-nt sequences instead of the naturally occurring intergenic sequences (Fig. 6).

We examined synthesis of genomic DI RNA and subgenomic DI RNA in DI RNA-transfected, MHV-A59-infected cells by Northern blot analysis using the ³²P-labeled *Sac*II–*Spe*I fragment of DE5-w3 as a probe (probe 2) (see Fig. 3). The molar ratio of genomic DI RNA to subgenomic DI RNA in the tested DI RNAs was approximately the same, except that FDI-6/7M synthesized less subgenomic DI RNA (Fig. 7). In spite of the significantly lower amounts of mRNA 2 and mRNA 3 relative to mRNA 7 that are inherent in MHV-infected cells, the 0.4-kb-long sequences flanking the intergenic region of genes 1–

2 and genes 2–3 demonstrated subgenomic DI RNA transcription activity that was similar to the 0.4-kb-long flanking sequences of the intergenic region of genes 6–7, indicating that no transcriptionally suppressive element existed in the naturally occurring flanking sequences adjacent to the intergenic regions at genes 1–2 and 2–3. Interestingly, sequence alteration from a wild-type intergenic sequence to the 12-nt sequence affected transcription efficiency only in the DI RNA containing the intergenic region from genes 6–7, but not in DI RNAs containing intergenic regions from genes 1–2 and 2–3. These data demonstrated that the sequence(s) surrounding the intergenic region of genes 6–7 contained a transcriptionally suppressive element(s) that suppressed transcription from the 12-nt sequence, but not from the naturally occurring 18-nucleotide-long intergenic sequence. Less subgenomic DI RNA synthesis in FDI-6/7M than in FDI-6/7wt was consistent with our previous study; in that study a mutant like FDI-6/7M, with the same deletion in the intergenic region between genes 6 and 7, synthesized significantly less subgenomic DI RNA than did a DI RNA with an intact intergenic region (Makino and Joo, 1993).

Sequences surrounding genomic cryptic consensus sequences suppressed transcription

MHV transcription regulation is flexible enough to recognize altered consensus sequences in which just one of seven consensus sequence nucleotides is changed to any of the three possible alternative bases (Joo and Makino, 1992). Indeed some MHV sequences that differ only by one nucleotide from the consensus sequence are transcriptionally active (Schaad and Baric, 1993). However, many genomic cryptic consensus sequences that also differ from UCUAAC by only one nucleotide do not act as sites for initiation of subgenomic RNA synthesis (Joo and Makino, 1992). Because some of the sequences flanking the inserted 12-nt sequence negatively affected subgenomic DI RNA transcription, subgenomic RNA transcription from a sequence that differs slightly from UCUAAC may possibly be suppressed by the sequences flanking that sequence; this could explain the flexibility of the consensus sequence.

To test this possibility we constructed a new series of mutant DI RNAs, all of which contained an insertion of a 0.4-kb fragment at the *Afl*II–*Sac*II site of DE5-w3. The inserted 0.4-kb fragment of FDI-M1, FDI-M2, and FDI-M3 corresponded to MHV-JHM sequences at about 0.7–1.1 kb, 7.9–8.3 kb, and 10.7–11.1 kb from the 5'-end, respectively. The center of these 0.4-kb fragments had a naturally occurring UCUAAC sequence, which differed by only one nucleotide from the UCUAAC consensus sequence. These naturally occurring UCUAAC sites in the MHV genome are not transcriptionally active. We also constructed FDI-M5 as a control clone; FDI-M5 was simi-

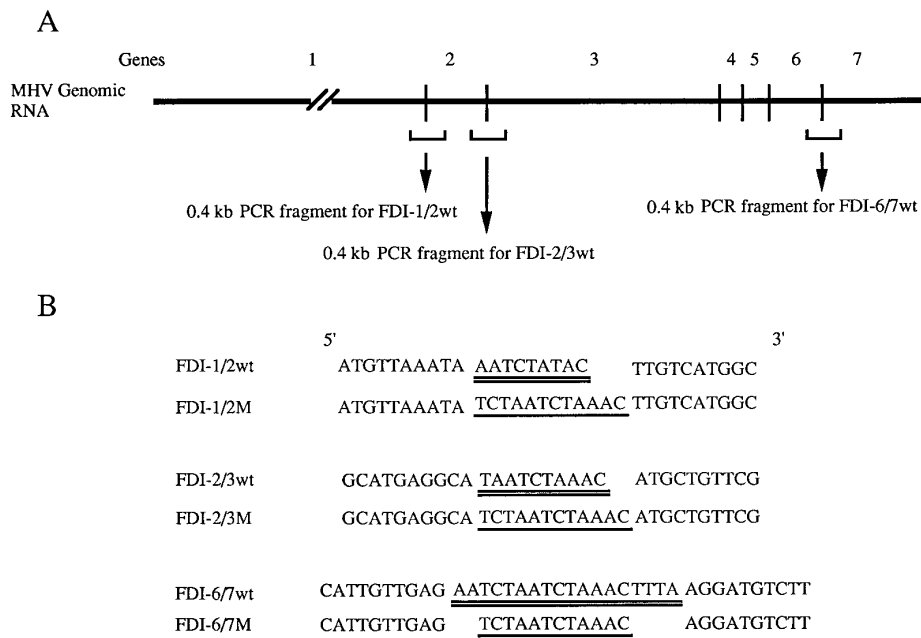


FIG. 6. Schematic diagram of the structure of the 0.4-kb fragment used for construction of FDI-1/2wt, FDI-2/3wt, and FDI-6/7wt. (A) Location of the 0.4-kb PCR fragments corresponding to the MHV genomic RNA. (B) Intergenic sequences and their flanking sequences of the 0.4-kb PCR fragments used for construction of FDI-1/2wt, FDI-2/3wt, and FDI-6/7wt and their mutants. Double lines show naturally occurring intergenic regions and single underlines represent the 12-nt sequence.

lar to FDI-1/2wt except that FDI-M5 contained a UCUUAAC sequence (Fig. 8) in place of the FDI-1/2wt AAUUAAC sequence (see Fig. 6B). FDI-M5 deleted the 5' two As of the intergenic sequence of FDI-1/2wt; FDI-M5 was a more appropriate control than FDI-1/2wt, because the presence of two As immediately upstream of the consensus sequence sometimes increases the level of subgenomic DI RNA transcription (Makino *et al.*, 1991).

We examined the synthesis of genomic DI RNA and subgenomic DI RNA in DI RNA-transfected, MHV-A59-infected cells by Northern blot analysis using the 32 P-labeled *Sac*I–*Spe*I fragment of DE5-w3 as a probe (probe 2, see Fig. 3). All the DI RNAs replicated efficiently, whereas subgenomic DI RNA synthesis occurred only in FDI-M5 (Fig. 9). Replication of FDI-M5 was lower than that of the other DI RNAs, which was consistent with our previous study showing that DI RNAs that do not synthesize subgenomic DI RNA replicate more efficiently than those that synthesize subgenomic DI RNA (Jeong and Makino, 1992). Here we clearly showed that flanking sequences of UCUUAAC in FDI-M1, FDI-M2, and FDI-M3 suppressed subgenomic DI RNA transcription, whereas the flanking sequences of UCUUAAC in FDI-M5 did not. Transcription from some of the genomic cryptic consensus sequences was suppressed by the sequences flanking these regions.

DISCUSSION

We have investigated whether different sequences flanking a 12-nt constructed sequence that carried an MHV transcription consensus region might affect subgenomic DI RNA transcription from that region. We looked at how location within the DI genome of the 12-nt sequence affected transcription. We checked whether naturally occurring flanking sequences of intergenic regions at genes 1–2, 2–3, and 6–7 affected transcription. Finally we studied whether naturally occurring flanking sequences of the genomic cryptic transcription consen-

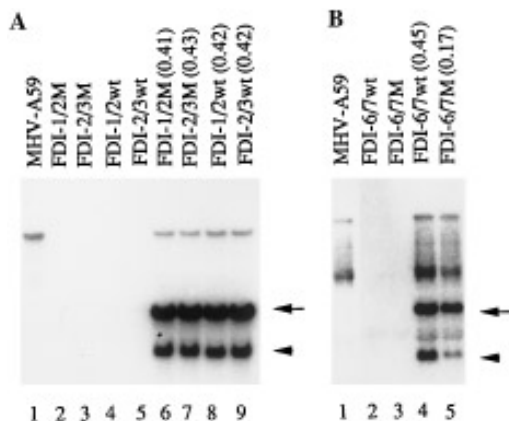


FIG. 7. Northern blot analysis of DI RNAs. Intracellular RNAs were extracted from DI RNA-transfected, MHV-infected cells (A, lanes 6–9; B, lanes 4 and 5) or DI RNA-transfected, mock-infected cells (A, lanes 2–5; B, lanes 2 and 3) and analyzed by Northern blot analysis. Lanes 1 of both panels represent RNA from MHV-infected cells. 32 P-labeled probe 2 (see Fig. 3) was used as a probe. Arrow and arrowhead represent genomic DI RNAs, subgenomic DI RNAs, respectively. The molar ratios of genomic DI RNA to subgenomic DI RNA are shown in parentheses.

ondary (or higher) structure of the internal cis-acting replication signal is indeed important for viral RNA synthesis (Kim and Makino, 1995b). The first set of DI RNAs which contained the 12-nt sequence within or proximal to these cis-acting replication signals replicated efficiently, indicating that the putative structures made by the cis-acting replication signals were probably maintained during DI RNA replication. To maintain active RNA structure for DI RNA replication, RNA structures formed by a 12-nucleotide sequence located within or near the predicted stable RNA structures of the cis-acting replication signal may not be transcriptionally optimal; the MHV transcription machinery may not have had easy access to a 12-nucleotide sequence located within a stable RNA structure, resulting in lower levels of subgenomic DI RNA synthesis.

FDI-6/7M synthesized a lower amount of subgenomic DI RNA than FDI-1/2M, and FDI-2/3M, indicating that flanking sequences of the intergenic sequence at genes 6–7 could suppress subgenomic DI RNA transcription. The presence of a transcription suppressive element(s) in the flanking sequences of the intergenic sequence at genes 6–7 was unexpected, because previous deletion analysis of this region did not reveal a transcription suppressive element (Makino and Joo, 1993). As shown in this study and previous studies (Makino *et al.*, 1991; Makino and Joo, 1993), if DI RNAs contained the natural occurring 18 nucleotide-long intergenic sequence, then the sequences flanking this region did not suppress subgenomic DI RNA transcription; the suppressive effect was only obvious when the intergenic region contained the 12-nt sequence. Most of DI RNAs we studied in our previous studies contained the 18-nucleotide-long naturally occurring intergenic sequence from genes 6–7 with its natural occurring flanking sequence, thus we could not detect the transcriptional suppressive effect of flanking sequences at genes 6–7 (Makino *et al.*, 1991; Makino and Joo, 1993).

For mutants FDI-1/2wt, FDI-1/2M, FDI-2/3wt, and FDI-2/3M that lacked the transcription suppressive element found in the flanking sequences, the sequence complementarily between the intergenic sequence and the 3'-end of leader sequence did not directly correlate with transcription efficiency. In a similar report, van der Most *et al.* (1994) concluded that the extent of base pairing between the leader RNA and the intergenic sequence does not control subgenomic RNA abundance; they analyzed several MHV-A59-derived DI RNAs with inserted transcription consensus sequences. On the contrary, decreasing complementarily between the intergenic sequence and the 3'-end of the leader sequence results in a decreasing level of subgenomic DI RNA transcription in DI RNAs with an inserted genes 6–7 region (Makino *et al.*, 1991; Makino and Joo, 1993). We speculate that the different conclusions between van der Most *et al.* (1994) and our previous studies probably reflect the dif-

ference in the flanking sequences of the inserted intergenic regions. Probably flanking sequences of the inserted transcription consensus sequence in the DI RNAs that were used by van der Most *et al.* (1994) do not contain a transcription suppressive element; in these DI RNAs minor changes in the intergenic sequence would not significantly affect transcription. We use DI RNAs with an inserted intergenic region from genes 6–7 that contained the transcription suppressive element; in these DI RNAs, when the intergenic sequence contained nucleotide deletions, the transcription suppressive element suppressed transcription. In yet another case, DI RNAs constructed to carry only the 18-nucleotide-long intergenic sequence of genes 6–7, show altered transcription efficiency when nucleotides within this sequence are deleted (Makino and Joo, 1993). In that study we chose the insertion site of the intergenic region arbitrarily, so that the sequences surrounding the inserted intergenic sequence possibly may have contained a transcription suppressive element(s); this is similar to the way that the sequence around the genes 6–7 intergenic sequence suppresses transcription. This may be the reason why synthesis of subgenomic DI RNA is susceptible to the sequence changes within the 18-nucleotide-long intergenic sequence in these DI RNAs.

The naturally occurring flanking sequences of the intergenic regions at genes 1–2 and 2–3 did not suppress subgenomic DI RNA transcription. The ns 30 protein and the S protein are translated from mRNA 2 and mRNA 3, respectively. The function of ns 30 is not clear, yet this is a conserved gene for many MHVs, except for one isolate that lacks most of this gene (Schwarz *et al.*, 1990). S protein is essential for MHV replication; S protein binds to the cell receptor to initiate infection (Dveksler *et al.*, 1991). Expression of both of those MHV genes seems to be crucial for replication and pathogenesis of MHV. That the flanking sequences of the intergenic regions of these genes did not suppress transcription indicated that transcriptionally suppressive sequences were eliminated during evolution of MHV. During the evolution of MHV, many mutations must have occurred within the sequences flanking the intergenic region of these genes; variant viruses whose flanking sequences did not suppress transcription might have had a selective advantage.

We showed that flanking sequences of the intergenic sequence at genes 6–7 contained the transcription suppressive element. MHV mRNA 7 synthesized from the intergenic region at genes 6–7 encodes the N protein that is essential for MHV replication; N protein forms helical nucleocapsid and is most likely required for viral RNA synthesis (Compton *et al.*, 1987; Kim and Makino, 1995a). Probably sequences that correspond to the transcription suppressive element encode an important function for either M protein or N protein, thus this transcription suppressive element was not eliminated during MHV

evolution; removal of such a sequence may significantly decrease MHV replication ability. Instead, MHV has evolved to contain the 18-nucleotide-long intergenic sequence that can overcome the transcription suppression effect from the flanking sequences.

One curious, unanswered question from this study is why the amount of mRNA 2 and mRNA 3 is significantly lower than mRNA 7, whereas the DI RNAs containing the 0.4-kb region from genes 1–2, 2–3, and 6–7 synthesized similar amounts of subgenomic DI RNA. This inconsistency may reflect the differences between the subgenomic DI system and actual MHV transcription. The major differences include the following: (1) The size of template RNA for subgenomic RNA synthesis; MHV genomic RNA is 31 kb, whereas genomic DI RNA is only 2.6 kb. (2) The locations of genes 1–2 and 2–3 are about 9.6 and 7.5 kb from the 3′-end of genome, respectively, whereas our experimental intergenic regions were located only 1.2 kb from the 3′-end of genomic DI RNA. (3) MHV genomic RNA has multiple intergenic regions, whereas genomic DI RNA had only one intergenic region.

How could these differences account for the disparity between the amounts of subgenomic DI RNA and real MHV mRNAs? Coronavirus RNA synthesis is considered to undergo nonprocessive transcription (Baric *et al.*, 1987; Makino *et al.*, 1986). Many nascent subgenomic mRNAs may fall off the template during RNA elongation, and some may not be able to resume transcription. If the length of the mRNA is longer, then the chance for premature subgenomic RNAs to detach from the template would be higher. We proposed that MHV transcription requires formation of a stable transcription complex that consists of the transcription consensus sequence, its flanking sequences and a putative scanning transcription factor which can migrate along negative-stranded RNA from the 5′-end toward the 3′-end (Joo and Makino, 1995). The transcription factor may dissociate from the template during scanning; if the length of the template is longer, the chances of the loss of a factor during scanning should be higher. Furthermore some scanning factors may dissociate from the template RNA after formation of the transcription complex. Indeed insertion of two intergenic regions, each of which is separated by about 125 nt, into DI RNA results in synthesis of two subgenomic DI RNAs, whereas the amount of the larger subgenomic RNA, which is transcribed from the upstream intergenic region (on the positive-sense RNA), is about 80% of the smaller subgenomic DI RNA (Joo and Makino, 1995). MHV genomic RNA contains many intergenic regions, many transcription scanning factors may fall off the template during scanning and this may result in the lower level of synthesis of the larger subgenomic mRNAs.

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