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Mechanism of Coronavirus Transcription: Duration of Primary Transcription Initiation Activity and Effects of Subgenomic RNA Transcription on RNA Replication

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Previously, we established a system whereby an intergenic region from mouse hepatitis virus (MHV) inserted into an MHV defective interfering (DI) RNA led to transcription of a subgenomic DI RNA in helper virus-infected cells. By using this system, the duration of a primary transcription initiation activity which transcribes subgenomic-size RNAs from the genomic-size RNA template in MHV-infected cells was examined. Efficient DI genomic and subgenomic RNA synthesis was observed when the DI RNA was transfected at 1, 3, 3.5, 5, and 6 h postinfection, indicating that all activities which are necessary for MHV RNA synthesis are present continuously during the first 6 h of infection. The effect of subgenomic DI RNA synthesis on DI genomic RNA replication was then examined. Replication efficiency of the DI genomic RNA which synthesized the subgenomic RNA was approximately 70% lower than that of DI genomic RNA which did not synthesize the subgenomic DI RNA in MHV-infected cells. Cotransfection of two different-size DI RNAs demonstrated that replication of the larger DI RNA was strongly inhibited by replication of the smaller genomic DI RNA. Cotransfection of two DI RNA species of the same length into MHV-infected cells demonstrated that reduced replication of the genomic DI RNA which synthesizes the subgenomic RNA did not affect the replication of cotransfected DI RNA, demonstrating that the reduction in DI genomic RNA replication works only in *cis*, not in *trans*. Therefore, the previously proposed hypothesis that coronavirus subgenomic RNA synthesis may inhibit the replication of genomic RNA by competing for a limited amount of virus-derived factors seems unlikely. Possible mechanisms of coronavirus transcription are discussed.

Mouse hepatitis virus (MHV), a coronavirus, is an enveloped virus containing a single-stranded, positive-sense RNA genome of approximately 31 kb (14, 15). In MHV-infected cells, seven to eight species of virus-specific subgenomic mRNAs comprising a 3′-coterminal nested-set structure (12, 17) are synthesized. These subgenomic mRNAs are named mRNAs 1 to 7, according to decreasing order of size (12, 17). mRNA 1 is structurally identical to the genomic RNA detected in MHV particles, whereas the other subgenomic mRNAs are not packaged into virions because of the lack of a packaging signal (27, 35). The 5′ end of the MHV genomic RNA contains a 72- to 77-nucleotide-long leader sequence (11, 13, 34). Downstream of the leader sequence are the MHV-specific genes, each of which is separated by a special short stretch of sequence, the intergenic sequence. A sequence identical to the leader sequence, of 72 to 77 nucleotides, is also found at the 5′ end of each MHV mRNA species, though these leader sequences are encoded for only once in the genomic strand. The leader sequences are fused with the mRNA body sequence which starts from the intergenic site consensus sequence of UCUAAAC (24, 33).

The mechanisms which produce this characteristic coronavirus mRNA structure are particularly intriguing. It has been proposed that coronavirus uses a unique form of leader RNA-primed transcription in which 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 (10). There is much experimental data to support this transcription model (1, 2, 10, 25). However, Sethna et al. demonstrated the presence of subgenomic-size, negative-strand RNAs containing the antileader sequence in coronavirus-infected cells (31, 32). In addition, subgenomic replicative intermediate (RI) RNAs corresponding to each MHV subgenomic mRNA species have been demonstrated in MHV-infected cells (30). In the leader RNA-primed transcription model, only a genomic-size negative-strand template is proposed to be used for subgenomic mRNAs; the presence of negative-strand subgenomic RNAs and subgenomic RI RNAs cannot be explained by this model. The presence of negative-strand subgenomic RNAs containing antileader sequence leads to the proposal that subgenomic mRNA synthesis may be involved in the replication of each subgenomic RNA species (31, 32). Furthermore, it was proposed that replicating subgenomic mRNAs may compete with the replicating genome for limiting factors required in RNA replication, possibly the RNA polymerase, in much the same way as do defective interfering (DI) RNAs (32). It should be noted that definitive evidence for any coronavirus subgenomic RNA amplification model has not yet been obtained.

We have previously established a system that exploits DI RNAs of MHV for deciphering the mechanism of coronavirus mRNA transcription (20). An MHV DIssF-derived complete cDNA clone containing an inserted intergenic region was constructed (20, 27). After transfection of the in vitro-synthesized DI RNA into MHV-infected cells, replication of DI genomic RNA as well as transcription of the DI subgenomic RNA were observed (20). This study clearly demonstrates that the initial subgenomic RNA transcription must undergo a step in which either the positive- or negative-sense genomic-size MHV RNA serves as the template for subgenomic RNA synthesis. Therefore, there are at least two

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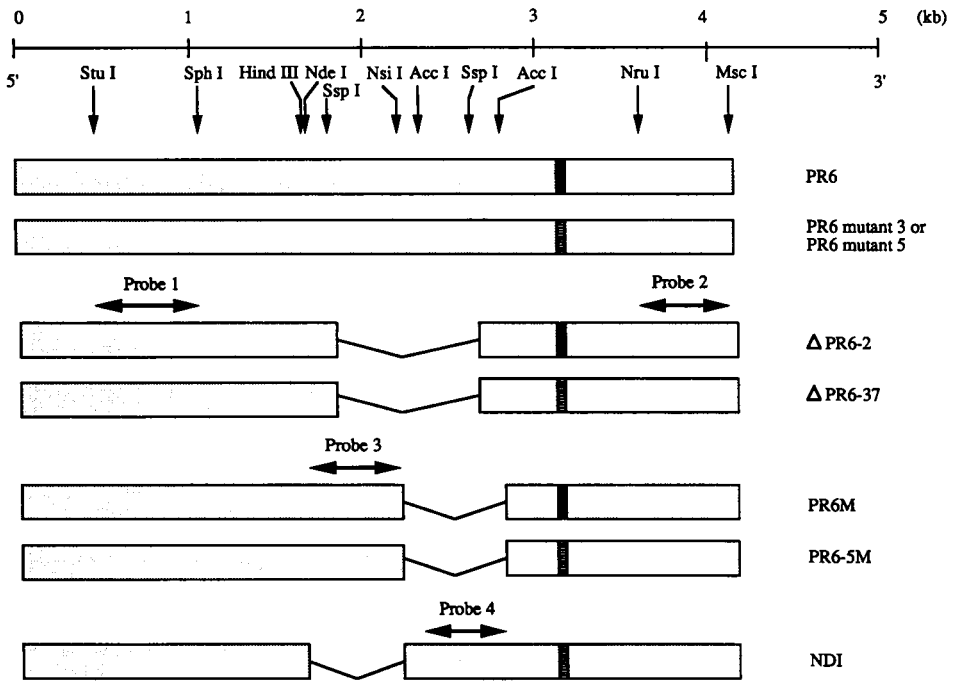

FIG. 1. Schematic diagram of the structure of DIssF-derived DI RNAs used in this study. The detailed structures of PR6, PR6 mutant 3, and PR6 mutant 5 were described previously (20). Black boxes and hatched boxes represent the intergenic regions derived from wild-type MHV-JHM and from PR6 mutant 3 or 5, respectively. No subgenomic DI RNA was synthesized at the intergenic regions shown by the hatched boxes. The shaded boxes represent the DI RNA genomic regions, and deleted regions are shown as thin lines. Probes 1 to 4 represent the DI cDNA regions used as probes in this study.

stages in coronavirus subgenomic RNA synthesis; we have named the first primary transcription, during which subgenomic-size RNA is made from the genomic-size template RNA, and we named the second secondary transcription, during which the subgenomic-size RNA is the template. For both transcription mechanisms, polarity of the template RNAs has not been conclusively demonstrated.

Although a number of in vitro coronavirus transcription or replication systems have been developed with the purpose of understanding coronavirus RNA synthetic mechanisms (3–6, 16, 18), there is no clear evidence for whether initiation of the primary transcription takes place in vitro. Recently, Baker and Lai reported the establishment of an in vitro coronavirus transcription system (1). In this system, in vitro-synthesized leader RNA species was exogenously added to cytoplasmic extracts obtained from MHV-infected cells, and it was observed that this exogenous leader sequence joined the 5′ end of subgenomic RNA species. This observation seems to suggest that initiation of primary transcription takes place in vitro, although the possibility that exogenous leader RNA binds to the antileader RNA sequence of previously synthesized subgenomic-size negative-strand RNA and subsequently elongates on those mRNAs cannot be excluded. Therefore, the duration of primary transcription initiation activity is not yet known.

In this study, two questions about coronavirus transcription were addressed. (i) When does the primary transcription initiation activity occur during the course of coronavirus replication? (ii) Does subgenomic RNA synthesis inhibit genomic RNA synthesis? The data are discussed in relation to the possible mechanism of coronavirus subgenomic RNA synthesis.

MATERIALS AND METHODS

Viruses and cells. The plaque-cloned A59 strain of MHV (MHV-A59) and the JHM strain (MHV-JHM) were used as helper viruses. Mouse DBT cells were used for propagation of viruses.

Plasmid construction. Plasmids ΔPR6-2 and ΔPR6-37 were constructed from PR6 and PR6 mutant 3 by the deletion of a 762-nucleotide *Ssp*I-*Ssp*I fragment located between nucleotides 1855 and 2617 (20, 27). Plasmids PR6M and PR6-5M were constructed by deleting a 544-nucleotide *Nsi*I-*Acc*I fragment between nucleotides 2222 and 2766 from the MHV DI cDNAs, PR6 and PR6 mutant 5 (20). Plasmid NDI was constructed from the MHV DI cDNA PR6 mutant 5 by deleting a 597-nucleotide *Hin*dIII-*Nsi*I fragment between nucleotides 1625 and 2222 (20). The structure of each mutant is depicted in Fig. 1.

RNA transcription and transfection. Plasmid DNAs were linearized by *Xba*I digestion and transcribed with T7 RNA polymerase as previously described (21). The lipofection procedure (7) was used for RNA transfection as previously described (20).

Preparation of virus-specific intracellular RNA and Northern (RNA) blotting. Virus-specific RNAs were extracted from MHV-infected cells as previously described (26). Intracellular RNA was denatured and electrophoresed through a 1% agarose gel containing formaldehyde and transferred to a nylon membrane as previously described (20). After baking, the membrane was prehybridized at 42°C for 1 h in prehybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 50 mM sodium phosphate

[pH 6.5], 0.1% sodium dodecyl sulfate [SDS], 50% formamide, 100 μg of sheared salmon sperm DNA per ml). The ^{32}P-labeled DNA probes were prepared by random-primed labeling of gel-purified MHV-specific cDNA fragments (28). Probe was denatured at 100°C for 10 min and added to the prehybridization solution, and hybridization was carried out at 42°C for 16 h. The membrane was washed three times at room temperature for 30 min and then twice at 50°C for 30 min in 2× SSC containing 0.1% SDS. The membrane was then air dried and exposed to Kodak XAR-5 film.

RESULTS

Longevity of the enzymatic activities required for MHV RNA synthesis in MHV-infected cells. To examine the duration of the primary transcription activity which initiates MHV subgenomic RNA transcription from the genomic-size RNA template, an MHV DI RNA encoding a transcribable subgenomic DI RNA was transfected into MHV-infected cells at different times postinfection (p.i.). The MHV DI RNA used in this study cannot replicate in the absence of helper MHV replication (20, 22, 27) and is completely dependent on helper virus-derived products for its transcription. If the initiation activity for the primary transcription is present only early in MHV replication, then DI subgenomic RNA synthesis should be observed only when DI genomic RNA is transfected early in infection. The in vitro-synthesized ΔPR6-2 DI RNA which can synthesize a subgenomic DI RNA in MHV-infected cells was used for this DI RNA transfection study. Equal amounts of in vitro-synthesized ΔPR6-2 DI RNA were transfected into MHV-JHM-infected DBT cells at 1, 3, or 5 h p.i. Virus-specific intracellular RNA species were extracted at 9 h p.i. and separated by agarose gel electrophoresis. Synthesis of the subgenomic DI RNA species was examined by Northern blotting analysis using an *Nru*I-*Msc*I MHV cDNA fragment, corresponding to nucleotides 18 to 352 from the 3′ end of MHV genomic RNA as a probe (probe 2; Fig. 1). In addition to the seven MHV-specific mRNA species, synthesis of a DI subgenomic RNA species of 1.1 kb was detected in MHV-infected cells which were transfected with in vitro-synthesized ΔPR6-2 DI RNA at 1, 3, and 5 h p.i. (Fig. 2a). Efficient replication of genomic DI RNA in MHV-infected cells was observed by Northern blot analysis using a probe specific for DI genomic RNA and MHV genomic RNA (probe 1; Fig. 1) (Fig. 2b). The same result was observed when PR6 DI RNA was used for transfection (data not shown). Although, as shown in Fig. 2, sometimes less DI genomic and subgenomic RNA was synthesized when transfection was at 1 h p.i. than after transfection at 3 and 5 h p.i., such differences were not always observed (Fig. 3). It is not known why DI RNA transfected at 1 h p.i. sometimes did not accumulate as well as did RNAs resulting from transfection at 3 and 5 h p.i. Efficient DI genomic RNA and subgenomic RNA synthesis was observed at 10 h p.i. in the MHV-A59-infected 17CL-1 cells which were transfected with ΔPR6-2 at 1, 3.5, or 6 h p.i. (data not shown). To confirm the data obtained from the Northern blot analysis, metabolic labeling of the subgenomic DI RNA species was performed. In vitro-synthesized ΔPR6-2 RNA was transfected into MHV-infected cells at 1 and 3 h p.i. After 1 h of incubation of RNA-transfected cells at 37°C, virus-specific RNA was labeled with $^{32}P_i$ in the presence of actinomycin D as previously described (26). After 1 h of labeling, virus-specific intracellular RNA species were extracted and separated by agarose gel electrophoresis. Synthesis of ^{32}P-labeled subgenomic DI RNA was demonstrated in both RNA preparations (data not shown). These results clearly demonstrated that all of the activities necessary for each step of MHV RNA synthesis exist continuously through the first 6 h of MHV replication.

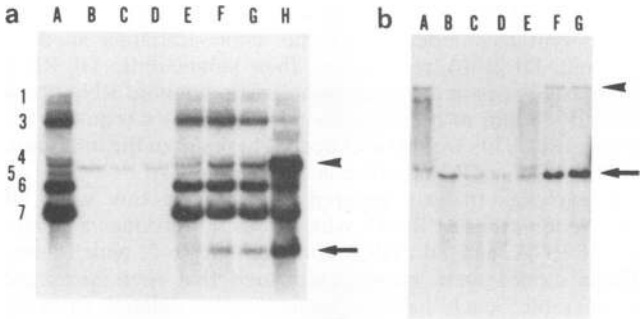

FIG. 2. Northern blot analysis of DI genomic and subgenomic RNAs synthesized in the DI RNA-transfected, MHV-infected cells. (a) The in vitro-synthesized ΔPR6-2 RNA was transfected into MHV-JHM-infected (lanes E to G) or mock-infected (lanes B to D) DBT cells at 1 (lanes B and E), 3 (lanes C and F), and 5 (lanes D and G) h p.i. Intracellular RNA was extracted at 9 h p.i., and virus-specific RNA species were separated on 1% formaldehyde gels and transferred to Biodyne nylon filters. MHV-specific RNA species were detected by using probe 2 (see Fig. 1). Lane A represents MHV-JHM-specific intracellular RNA species; lane H represents the MHV-specific intracellular RNA species obtained after infection with the virus inoculum which was obtained after one passage of the virus sample from ΔPR6-2-transfected, MHV-JHM-infected cells. Numbers 1 to 7 represent the MHV-JHM-specific mRNA species. The arrow and arrowhead represent DI subgenomic RNA and DI genomic RNA, respectively. (b) The intracellular RNA samples shown in panel a were analyzed by using probe 1. The order of samples is the same as in panel a, with the omission of lane H. DI genomic RNA and MHV-JHM mRNA 1 are represented by the arrow and arrowhead, respectively.

Effects of subgenomic DI RNA transcription on the accumulation of genomic DI RNA. To examine whether subgenomic RNA synthesis inhibits the replication of genomic RNA by competing for a limited amount of helper virus-derived factor, the replication efficiencies of two DI genomic RNAs, one of which synthesizes a subgenomic RNA and the

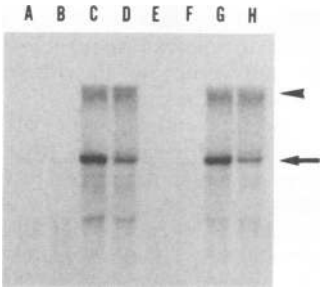

FIG. 3. Northern blot analysis of ΔPR6-37 and ΔPR6-2 DI genomic RNAs in DI RNA-transfected, MHV-A59-infected cells. Equal amounts of in vitro-synthesized ΔPR6-2 RNA (lanes B, D, F, and H) and ΔPR6-37 RNA (lanes A, C, E, and G) were transfected into MHV-A59-infected (lanes C, D, G, and H) or mock-infected (lanes A, B, E, and F) DBT cells at 1 (lanes E to H) or 3 (lanes A to D) h p.i. Intracellular RNA was extracted at 7 h p.i., and virus-specific RNA species were separated on 1% formaldehyde gels and transferred to Biodyne nylon filters. MHV-A59 mRNA 1 (arrowhead) and the DI genomic RNAs (arrow) were detected by using probe 1 (see Fig. 1).

other of which does not, were compared. If subgenomic DI RNA synthesis indeed uses the same activities as does genomic DI RNA replication, then subgenomic DI RNA synthesis is predicted to compete with genomic RNA for a limited amount of helper virus-derived factors requisite for replication. This would be expected to result in the inhibition of DI genomic RNA synthesis.

The clones to be compared after transfection were DI cDNA construct ΔPR6-37, which does not produce a subgenomic RNA, and DI cDNA construct ΔPR6-2, which does. These clones were constructed such that their sizes are comparable; each has the same region deleted from its parent. The parental strains differ slightly at their intergenic regions, which confers the difference in regulation of subgenomic RNA synthesis (20).

Equal amounts of in vitro-synthesized ΔPR6-2 and ΔPR6-37 DI RNAs were individually transfected into MHV-A59-infected DBT cells at 1 or 3 h p.i. and at 7 h p.i., intracellular RNA species were extracted, and equal amounts of intracellular RNAs were electrophoresed on a 1% agarose gel. The replication of DI genomic RNA was detected by Northern blot analysis using a probe specific for the region corresponding to nucleotides 482 to 1088 from the 5′ end of MHV DI RNA, DIssF (probe 1; Fig. 1) (27); only MHV genomic RNA and DI genomic RNA are detectable by this probe. It was evident that both DI RNAs replicated efficiently in MHV-infected cells regardless of the time of transfection, yet the amount of replicating ΔPR6-2 was less than that of ΔPR6-37 (Fig. 3). This difference was consistently observed, without exception, in five independent experiments. The amount of replicating DI genomic RNA was examined by densitometric scanning of the autoradiograms, and it was found that the accumulation of ΔPR6-2 replication averaged 70% less than that of ΔPR6-37. A similar result was obtained when replication of the PR6 DI genomic RNA and replication of PR6 mutant 5, with differing intergenic sites, were compared in MHV-infected cells (data not shown), indicating that the less efficient accumulation of DI RNA seen in ΔPR6-2, which synthesizes a subgenomic RNA, was not unique to this particular DI RNA but was probably an effect of subgenomic RNA synthesis. Also, the sequence alterations at the intergenic regions of PR6 mutant 5 and ΔPR6-37, which both result in a loss of subgenomic RNA synthesis, are different mutations (20). That again points to the probability that lowered accumulation of those DI genomic RNAs which also synthesize subgenomic DI RNA was due not to specific sequence deletions at the intergenic region but rather to the inhibitory effects of subgenomic DI RNA synthesis.

Though less efficient replication of DI genomic RNA encoding transcribable subgenomic DI RNA was observed, the difference between accumulation of ΔPR6-2 and ΔPR6-37 genomic DI RNA was not great. Next, the effect on replication efficiency of ΔPR6-2 genomic DI RNA by another, smaller DI genomic RNA, DIssE of 2.3-kb length (19, 21, 23), was tested. It has been generally thought that smaller DI RNAs can replicate more efficiently than larger DI RNAs in a given time, and as a result, smaller DI RNAs can interfere with the replication of helper virus more efficiently than can larger DI RNAs (8). Since DIssE RNA requires helper virus infection for its replication, it probably shares enzymatic activities for its replication with ΔPR6-2 (22). If the reduced replication of the subgenomic DI RNA-producing genomic DI RNA is due to the competition for a limited amount of helper virus-derived virus factors between the 1.1-kb subgenomic DI RNA and the 3.4-kb DI genomic RNA, then the replication of the 2.3-kb DIssE RNA would be expected to have the same or a smaller effect on the accumulation of ΔPR6-2 genomic DI RNA. A 70% inhibition of ΔPR6-2 genomic DI RNA by the replication of DIssE would suggest that the proposed hypothesis is correct.

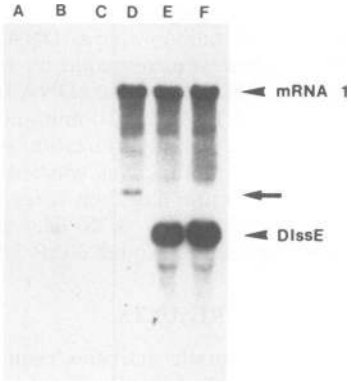

FIG. 4. Northern blot analysis of ΔPR6-2 and DE5-w3 DI genomic RNAs in DI RNA-transfected, MHV-A59-infected cells. The in vitro-synthesized ΔPR6-2 RNA, DE5-w3 RNA (21), and a 2.3-kb-long irrelevant RNA species containing the pT7-4 sequence (21) were prepared, and equal amounts of the RNAs were mixed in the transfection buffer. The mixtures of ΔPR6-2 and the irrelevant 2.3-kb RNA (lanes A and D), ΔPR6-2 and DE5-w3 (lanes B and E), and DE5-w3 and the irrelevant 2.3-kb RNA (lanes C and F) were transfected into the MHV-A59-infected (lanes D to F) or mock-infected (lanes A to C) cells at 3 h p.i. Virus-specific RNA species were extracted at 7 h p.i., separated on 1% formaldehyde gels, and transferred to Biodyne nylon filters. The probe was probe 1 (see Fig. 1). The arrow represents the genomic RNA of ΔPR6-2. The mRNA 1 of MHV-A59 and the genomic RNA of DE5-w3 are shown as mRNA 1 and DIssE, respectively.

To examine how efficiently DIssE replication inhibits the replication of ΔPR6-2, the same amounts of in vitro-synthesized DIssE-specific RNA, DE5-w3 DI RNA (21), and ΔPR6-2 DI RNA were mixed in the lipofection solution and then transfected into MHV-A59-infected cells at 3 h p.i., and the intracellular virus-specific RNA was extracted 7 h p.i. As a control, equal amounts of DE5-w3 RNA and ΔPR6-2 DI RNA were mixed with equal amounts of irrelevant in vitro-synthesized RNA species and individually transfected into MHV-infected cells. Levels of DI genomic RNA replication were estimated by Northern blot analysis using probe 1, which specifically hybridizes to DI genomic RNA and MHV genomic RNA (Fig. 4). Efficient replication of DE5-w3 DI RNA was observed in the DE5-w3 DI RNA-transfected cells, and the amount of the accumulated DE5-w3 DI RNA was approximately 40 times higher than that of ΔPR6-2 DI RNA (Fig. 4, lanes D and F). The replication of ΔPR6-2 was strongly inhibited by the replication of DE5-w3 DI RNA (lane E). Densitometric scanning analysis demonstrated that the inhibition of ΔPR6-2 DI RNA replication in DE5-w3-replicating cells was at least 96%. Replication of ΔPR6-37 DI genomic RNA was also strongly inhibited at the same extent by cotransfection of the DE5-w3 DI RNA (data not shown). These results clearly demonstrated that two different RNA molecules were cointroduced into MHV-infected cells by the lipofection procedure and that the smaller DIssE RNA can strongly inhibit replication of the larger DIssF-derived DI RNA.

Inhibition of genomic DI RNA replication resulting from subgenomic DI RNA synthesis occurs only in *cis* orientation.

The data presented above suggested that the mechanism(s) which caused the reduced replication of genomic DI RNA in the DI encoding a subgenomic RNA was different from the mechanism(s) which caused inhibition of genomic DI RNA by the smaller DIssE RNA. This finding led to the hypothesis that the limited reduction in ΔPR6-2 DI genomic RNA replication shown in Fig. 3 was not due to a competitive effect but perhaps was the result of yet another mechanism(s). If this hypothesis is correct, then such an inhibitory effect on the replication of DI genomic RNA which synthesizes the DI subgenomic RNA probably works only in *cis*, not in *trans*. To test this hypothesis, three DI cDNAs, NDI, PR6M, and PR6-5M, were constructed (Fig. 1). NDI and PR6-5M were derived from PR6 mutant 5, so that no subgenomic DI RNA was synthesized from these DI genomic RNAs, whereas PR6M derived from PR6 did synthesize a subgenomic RNA species. PR6M and PR6-5M were deleted from nucleotides 2222 to 2792 at the 5′ ends of their parental DI cDNAs, and NDI was deleted from nucleotides 1625 to 2222 at the 5′ end of PR6 mutant 5 cDNA. These three in vitro-synthesized DI RNAs are very similar in size, and the replication of NDI can be distinguishable from that of PR6M or PR6-5M by simply choosing a specific cDNA probe (Fig. 1).

To examine whether the effect of reduced replication works in *trans*, equal amounts of in vitro-synthesized NDI, PR6M, and PR6-5M were prepared. Mixtures of PR6M and NDI and of PR6-5M and NDI were transfected into MHV-A59-infected cells at 3 h p.i. At 7 h p.i., intracellular RNA was extracted and equal amounts of RNA were applied to agarose gels. The amount of DI RNA replication was then examined by Northern blot analysis. Efficient replication of PR6M and PR6-5M was detected in MHV-A59-infected cells by using probe 3, the *Nde*I-*Nsi*I fragment of PR6 MHV DI cDNA that specifically binds to MHV genomic RNA and to PR6M and PR6-5M genomic DI RNA but not to NDI genomic DI RNA. Accumulation of PR6M genomic RNA, which synthesizes subgenomic DI RNA, was about 80% lower than that of PR6-5M in NDI-cotransfected cells (Fig. 5b, lanes I and J). This observation was consistent with the observation described above. In contrast to this finding, there was no detectable difference between the accumulation of NDI in PR6M replicating cells and in PR6-5M replicating cells when the *Acc*I-*Acc*I fragment of MHV cDNA was used as a probe (probe 4; Fig. 1) (Fig. 5a, lanes H and I). The same conclusion, based on three independent experiments, was that the limited reduction in replication resulting from subgenomic DI RNA synthesis worked in the *cis* but not in the *trans* configuration.

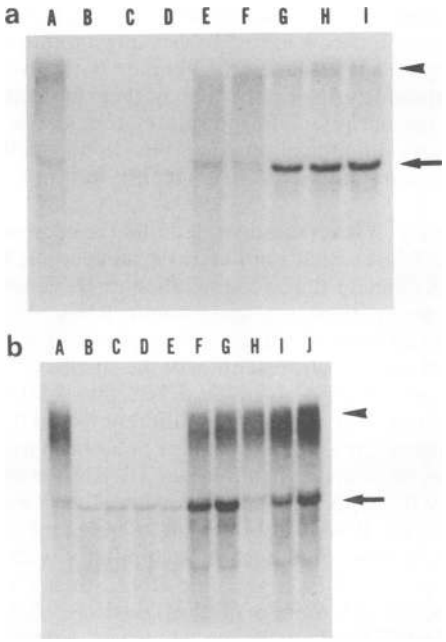

FIG. 5. Northern blot analysis of PR6M, PR6-5M, and NDI genomic RNAs in DI RNA-transfected, MHV-A59-infected cells. In vitro-synthesized PR6M, PR6-5M, and NDI RNAs and a 3.6-kb-long irrelevant RNA species containing pT7-4 sequence plus a 2.0-kb-long lambda *Hin*dIII-*Hin*dIII fragment were prepared, and equal amounts of the RNAs were mixed in the transfection buffer. The mixtures of RNA samples were transfected into MHV-A59-infected or mock-infected cells at 3 h p.i. Virus-specific RNA species were extracted at 7 h p.i., separated on 1% formaldehyde gels, and transferred to Biodyne nylon filters. Probes 4 and 3 were used for detecting NDI genomic RNA (a) and PR6M or PR6-5M DI genomic RNA (b), respectively. (a) Mixtures of NDI and the 3.6-kb RNA (lanes B and G), NDI and PR6M (lanes C and H), NDI and PR6-5M (lanes D and I), PR6M and the 3.6-kb RNA (lane E), and PR6-5M and the 3.6-kb RNA (lane F) were transfected into MHV-A59-infected (lanes E to I) or mock-infected (lanes B to D) cells. Lane A represents RNA species in MHV-A59-infected cells. The arrow and arrowhead represent NDI genomic RNA and MHV-A59 mRNA 1, respectively. (b) Mixtures of PR6M and the 3.6-kb RNA (lanes B and F), PR6-5M and the 3.6-kb RNA (lanes C and G), NDI and PR6M (lanes D and I), NDI and PR6-5M (lanes E and J), and NDI and the 3.6-kb RNA (lane H) were transfected into MHV-A59-infected (lanes F to J) or mock-infected (lanes B to E) cells. Lane A represents RNA species in MHV-A59-infected cells. The arrow and arrowhead represent DI genomic RNA and MHV-A59 mRNA 1, respectively.

DISCUSSION

Primary transcription initiation activity existed continuously during at least the first 6 h of MHV RNA synthesis. In this study, it was unambiguously demonstrated that MHV RNA synthesis activities, including primary transcription and secondary transcription, were continuously present from 1 to at least 6 h p.i. These results strongly indicate that positive- or negative-sense genomic RNA synthesized later in infection not only is used for subsequent RNA replication but also serves as a template for the initiation of primary transcription. The results obtained from this study were not consistent with the observation reported by Brayton et al. (3, 4) that an activity producing the negative-strand genomic RNA is present only early in infection but are consistent with the observation made by Sawicki and Sawicki (29) in which the rate of overall negative-strand RNA synthesis increases until 5 to 6 h p.i. in MHV-infected cells. Probably this genomic-size negative-strand RNA continuously synthesized from 1 to 6 h p.i. is biologically functional.

Mechanism of coronavirus subgenomic RNA synthesis. From the discovery of the subgenomic negative-strand RNA species, it was proposed that coronavirus mRNA undergoes an amplification process which might inhibit the replication of genomic RNA by competing for a limited amount of virus-derived factors, including RNA polymerases (32). Identification of the subgenomic-size RI RNA species in MHV-infected cells demonstrated that indeed there must be elongation of RNA molecules on the subgenomic-size RNA templates (30). Primary transcription is considered to be that

which initiates synthesis on genomic-size RNA, and secondary transcription is considered to use subgenomic-size RNA as the template. It is not clear whether both positive- and negative-strand RNAs or either one of them elongates during secondary transcription. Furthermore, conclusive evidence confirming the amplification (or replication) mechanism of subgenomic coronavirus RNA species has not yet been obtained.

In this study, it was demonstrated that the accumulation of DI genomic RNA which synthesizes a subgenomic RNA was slightly less efficient than accumulation of DI genomic RNA that does not synthesize a subgenomic RNA. Also, it was shown that this limited reduction in replication worked only in *cis*, not in *trans*. Cotransfection of the smaller DIssE RNA with a larger DIssF-derived DI RNA into MHV-infected cells indicated that when two different-size DI genomic RNAs compete for a limited amount of helper virus-derived factors, the replication of the larger DI RNA was strongly inhibited by the replication of the smaller DIssE (Fig. 4). The subgenomic DI RNA is about half the size of the DIssE RNA; thus, if subgenomic RNA accumulates with the same efficiency as does DIssE genomic RNA, then more of the subgenomic DI RNA than of the DIssE RNA should be produced in MHV-infected cells. However, accumulation of DIssE was at least 40 times higher than that of the subgenomic RNA species even though equal amounts of DIssE RNA and ΔPR6-2 were individually transfected (20) (Fig. 4). These observations did not support the hypothesis proposed by Sethna et al. in which it is suggested that subgenomic RNA amplification may inhibit the replication of genomic RNA by competing for limited virus-derived RNA polymerase (32).

From the data obtained in this study and those obtained previously by us and others, the following different coronavirus transcription models are considered.

In the first of these models, coronavirus subgenomic RNA secondary transcription may undergo its amplification step at a reduced efficiency while sharing the same activities which are used for genomic RNA replication. Our data indicated that the accumulation of subgenomic DI RNA was much less efficient than that of DIssE RNA. Therefore, if subgenomic RNAs are amplified via a mechanism which shares RNA replication machinery, it is amplified, unexpectedly, at a reduced efficiency. This reduced efficiency would suggest a lack of competition with genomic RNA replication. Perhaps the low efficiency of subgenomic DI RNA amplification is due to the structure at its positive-sense 5′ region; it contains the complete leader sequence at the 5′ end but lacks a region downstream of the leader sequence which is present on the genomic RNA. In this model, the limited reduction in the genomic DI RNA accumulation cannot be due to competition for helper virus-derived factors; it must be due to another, yet unknown mechanism, because the effect of reduced replication worked in *cis* but not in *trans*. One possible explanation is that during the time that a certain percentage of DI genomic-size RNAs are functioning as templates for primary transcription, they cannot be used for genomic RNA replication, at least temporarily.

In a second model, it is possible that secondary transcription undergoes the amplification step via a transcriptional activity which differs from genomic RNA replication activity; thus, competition of virus-derived factors would not be a factor. This mechanism, again, would account for the lower efficiency of subgenomic RNA amplification relative to replicative functions, and the limited reduction in DI genomic RNA accumulation seen in those DIs synthesizing subgenomic RNA may be explained as discussed above.

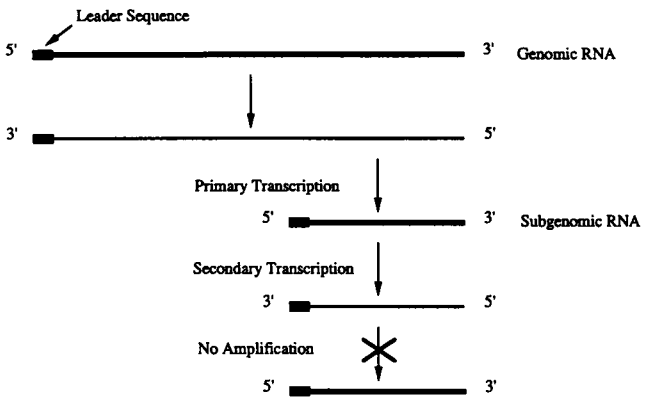

FIG. 6. Schematic illustration of a new coronavirus transcription model. Positive- and negative-strand RNAs are shown by bold and thin lines, respectively. Only one subgenomic RNA species is illustrated for schematic simplicity.

If secondary transcription involves an amplification step and if the efficiencies of degradation of each subgenomic mRNA species are the same, then it is reasonable to hypothesize that the molar ratio of each subgenomic RNA may vary during the course of infection, because smaller subgenomic RNAs should accumulate more efficiently than larger mRNA species. Furthermore, larger mRNAs would be expected to degrade more efficiently than smaller ones. If this is the case, an even more efficient accumulation of smaller subgenomic RNA species would be expected. However, it was demonstrated that the ratio of the amount of each subgenomic RNA is essentially constant during MHV replication (17). If primary transcription is short-lived, then it is quite reasonable to speculate that the subgenomic RNA accumulates by an amplification mechanism. However, as is demonstrated in this study, primary transcription takes place continuously during the first 6 h of MHV RNA synthesis. Considering these points, we propose a third model for coronavirus transcription (Fig. 6).

This new model proposes that secondary transcription is not an amplification step but is involved only in the synthesis of negative-strand subgenomic RNA from the subgenomic positive-strand RNA which was synthesized during primary transcription. The synthesized negative-strand subgenomic RNA does not undergo further amplification but results, in this model, in dead-end products. Only a negative-strand RNA elongates on the subgenomic RI RNA. Because the 3′-end regions of the positive-strand subgenomic mRNAs and the positive-strand genomic RNA are identical structures, it is reasonable to hypothesize that the negative-strand subgenomic RNA is copied from the positive-strand subgenomic mRNA with almost the same efficiency as genomic-size negative-strand RNA synthesis. Because of the lack of other essential sequences at the 5′ region of the positive-strand subgenomic RNA, positive-strand subgenomic RNA is not synthesized from the negative-strand subgenomic RNAs. This model is consistent with most previously described observations; subgenomic DI RNA synthesis does not occur in subgenomic DI RNA-transfected, MHV-infected cells (20), subgenomic RI RNAs are present in MHV-infected cells (30), the molar ratio of each subgenomic replicative-form (RF) RNA is parallel to that of subgenomic

mRNA species (30), and antileader sequences are present on negative-strand subgenomic RNA (31). This model also agrees with our observation that primary transcription is continuous during at least the first 6 h of MHV RNA synthesis. This finding suggests that continuously synthesized positive-strand subgenomic RNA made by a primary transcription mechanism is continuously available as a template for the negative-strand subgenomic RNA species. Furthermore, we have recently observed that the negative-strand subgenomic DI RNA was indeed synthesized after transfection of in vitro-synthesized positive-strand subgenomic DI RNA into MHV-infected cells (9). This observation strongly supports this model.

Sawicki and Sawicki (30) proposed a different model to explain coronavirus transcription. In their model, the subgenomic negative-strand RNAs are initially synthesized from the input genomic RNA (primary transcription). Then, the positive-strand subgenomic RNA is synthesized on the subgenomic-sized negative-stranded RNA (secondary transcription) (30). This model is also consistent with the bulk of observations except that the genome-length RI RNA does not produce the subgenomic-size RF RNAs after RNase digestion (30). If Sawicki and Sawicki's model is correct, then why are subgenomic-size RF RNAs not produced after RNase treatment of the genomic-size RI RNA that is elongating subgenomic negative-strand RNA species on the genomic-size positive-sense RNA? From this study and our previous study, it is evident that the subgenomic RNA must be initially synthesized from the genomic-size RNA (20) and that this primary transcription continues from 1 to at least 6 h p.i. Therefore, any transcription model should propose that subgenomic RF RNAs be generated after RNase digestion of the genomic-size RI RNA. At present, it is not clear why subgenomic RF RNAs are not produced from genome-length RI RNA.

In these studies using the MHV DI cDNA system, it was unambiguously demonstrated that coronavirus transcription is at least a two-step process involving primary transcription and secondary transcription. As discussed above, a number of different models of MHV transcription can be considered. This study and studies now in progress to unravel the mechanism of subgenomic RNA synthesis through the investigation of MHV DI cDNA clones should prove to be the key to understanding the coronavirus transcription mechanism.

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