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Importance of coronavirus negative-strand genomic RNA synthesis prior to subgenomic RNA transcription

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Abstract

The (−)-strand viral RNAs that result from after infection of cells with coronaviruses, which possess RNA genomes of message polarity, are genomic-sized and subgenomic-sized. Each of the (−)-strand subgenomic RNAs corresponds in size to each of the subgenomic mRNA species that are made in infected cells. We tested whether (−)-strand subgenomic RNAs might initially be synthesized from the input single-stranded (+)-strand genomic RNA prior to the production of subgenomic mRNAs. We used a mouse hepatitis virus (MHV) defective interfering (DI) RNA, from which subgenomic RNA was produced in DI RNA-replicating cells, because this DI RNA had a functional MHV intergenic region inserted in its interior. MHV samples containing the DI particles were irradiated with UV-light and then superinfected into cells that had been infected with MHV 4 h prior to superinfection. Northern blot analysis of intracellular RNAs that were extracted 3 h after superinfection showed that genomic DI RNA and subgenomic DI RNA had similar UV-target sizes, indicating that (−)-strand genomic DI RNA synthesis from input genomic DI RNA probably occurred prior to the subgenomic-size DI RNA synthesis. We discuss why, in the course of coronavirus transcription, (−)-strand genomic-length coronavirus RNA synthesis might occur before subgenomic-sized RNAs of either polarity are made.

Keywords: Coronavirus; RNA transcription; Negative-strand RNA

Mouse hepatitis virus (MHV), a prototypic coronavirus, contains a 31-kb long, single-strand genome made up of (+)-sense RNA (Lai and Stohlman, 1978; Pachuk et al., 1989; Lee et al., 1991; Bonilla et al., 1994). The 5′-end of MHV genomic RNA contains a 72–77-nucleotide long leader sequence. The MHV-specific genes, which are all located downstream from the leader, are separated from one another by an intergenic region. MHV-infected cells produce genomic-size mRNA and six to seven species of subgenomic

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mRNA species that make up a 3′-coterminal nested group, in which each mRNA carries one more gene in the 5′ direction than the last (Lai et al., 1981; Leibowitz et al., 1981). All these virus-specific mRNAs have a leader RNA sequence at their 5′-end (Lai et al., 1983, 1984; Spaan et al., 1983) and a poly (A) tail at the 3′-end. On the subgenomic mRNAs, the leader sequence is fused to the intergenic region (Spaan et al., 1983; Lai et al., 1984). Genomic-size and subgenomic-size (−)-strand RNAs, each of them corresponding in size to each of the subgenomic mRNA species, are also present in coronavirus-infected cells (Sethna et al., 1989). These (−)-strand RNAs contain an antileader sequence at the 3′-end and poly U sequence at the 5′-end (Sethna et al., 1991).

Several models have been put forward to explain the progression of coronavirus (−)-strand subgenomic RNA synthesis. Sawicki and Sawicki (1990) proposed that (−)-strand subgenomic RNAs are synthesized from (+)-strand genomic RNA and serve as templates for subgenomic mRNA synthesis. They speculated that (−)-strand RNA synthesis terminated at the intergenic regions to produce (−)-strand subgenomic RNAs. Another possibility is that (−)-strand subgenomic RNAs may be synthesized by processing of precursor (−)-strand genomic RNAs that are synthesized from (+)-strand genomic RNA. Other models propose that subgenomic mRNAs are synthesized prior to production of (−)-strand subgenomic RNAs. In these models, subgenomic mRNAs may result from the processing of (+)-strand genomic RNA, from transcription off (−)-strand genomic RNA by a unique leader-primed transcription mechanism, in which short 'free' leader RNAs serve as primers for subgenomic mRNA synthesis (Baric et al., 1983), or from polymerase jumping off the leader sequence to an intergenic region during subgenomic mRNA synthesis (Spaan et al., 1983). The (−)-strand subgenomic RNAs would then be copied from the subgenomic mRNAs, and might be active templates for subgenomic mRNA synthesis (Sethna et al., 1989; Sawicki and Sawicki, 1990; Schaad and Baric, 1994) or dead-end transcription products (Jeong and Makino, 1992).

Here, we indirectly tested the possibility that (−)-strand subgenomic RNAs are synthesized from the full-length (+)-strand genomic RNA prior to subgenomic mRNA synthesis. For these experiments, we chose a genetically altered MHV defective interfering (DI) particle that makes a subgenomic RNA; this DI has an added intergenic region placed internally, from which subgenomic DI RNA synthesis is initiated in DI RNA-replicating cells (Makino et al., 1991). An MHV sample containing the DI particles was irradiated with UV-light, and then used to superinfect cells that had been infected with MHV 3 h earlier. Intracellular RNAs were extracted 4 h after superinfection, and the relative amounts of genomic DI RNA and subgenomic DI RNA were examined by using Northern blot analysis. The rationale behind this experimental approach is as follows:

(i) UV-irradiation of DI particles induces UV-lesions on genomic DI RNA. This should result in the inhibition of elongation of (−)-strand DI RNA at the UV-lesion. If (−)-strand subgenomic DI RNAs are synthesized from the input (+)-strand genomic DI RNA, then synthesis of (−)-strand subgenomic DI RNA is most likely more resistant to UV-irradiation than the synthesis of (−)-strand genomic DI RNA. If (−)-strand subgenomic DI RNAs are synthesized prior to subgenomic DI RNA synthesis, then (−)-strand subgenomic DI RNA is most probably used as template for subgenomic DI RNA synthesis. The synthesis of (+)-strand subgenomic DI RNA should also be more resistant to damage by UV-light than the synthesis of genomic DI RNA, because the UV-target size of subgenomic DI RNA should be smaller than that of genomic DI RNA.

(ii) Subgenomic DI RNA synthesis appears not to require DI RNA replication. In MHV-infected cells, expression (Liao and Lai, 1994) or transfection (Lin et al., 1994, 1996) of a (+)-strand MHV DI RNA fragment that contains an inserted intergenic region and that lacks one part of the DI RNA *cis*-acting replication signals (Kim et al., 1993; Kim and Makino, 1995; Lin and Lai, 1993) results in transcription of subgenomic DI RNA. These DI RNA fragments synthesize (−)-strand

RNAs, yet do not undergo DI RNA replication (Lin et al., 1994). We can, therefore, assume that much of the input UV-irradiated genomic DI RNA should proceed to the transcription step without undergoing a DI RNA replication step.

(iii) Helper virus-derived activities that are necessary for DI RNA replication and transcription were immediately available after superinfection with the UV-irradiated DI particles.

(iv) As shown below, replication and transcription of superinfecting MHV DI RNA was not suppressed by the initial infectant, MHV-A59.

We constructed an MHV DI cDNA, MIGCAT, which consisted of three distinct regions (Fig. 1). The 5′-most, 3.1-kb long region of MIGCAT was made from the corresponding region of a cloned MHV-JHM DIssF DI RNA (Makino et al., 1990), PR6 (Makino et al., 1991). The second region contained an intergenic sequence (5′-AAUCUAAUCUAAACUUUA-3′) and the 5′-most, 0.27-kb region of the chloramphenicol acetyltransferase (CAT) gene. The most 3′-end region of MIGCAT came from the very 3′-end of the 0.46-kb long MHV-JHM genomic RNA. MIGCAT DI RNA contained MHV-JHM DI RNA *cis*-acting replication signals (Kim et al., 1993; Kim and Makino, 1995; Lin and Lai, 1993), a packaging signal (van der Most et al., 1991; Fosmire et al., 1992; Woo et al., 1997), and an intergenic sequence. We expected MIGCAT DI RNA to replicate and transcribe subgenomic DI RNA in MHV-infected cells. MIGCAT DI RNA transcripts were synthesized in vitro using T7 polymerase (Makino and Lai, 1989), and then transfected into MHV–A59-infected DBT cells (Hirano et al., 1974) using a lipofection procedure, as described previously (Makino et al., 1991). Virus released into the culture supernatant was collected 15 h after RNA transfection and was subsequently passaged twice on DBT cells to amplify the DI particles containing MIGCAT DI RNA (MIGCAT DI particles). We used this virus preparation, which is designated P2, in this study. The presence of MIGCAT DI RNA was confirmed by Northern blot analysis of virion RNA extracted from the gradient-purified P2 virus sample using CAT-specific probe (data not shown).

To ensure that the majority of the input MIGCAT DI RNA would be transcribed immediately, we wanted to superinfect MIGCAT DI particles into MHV-infected cells when MHV transcription activities were active. For determining the start of MHV transcription activities in MHV-infected cells, we coinfected DBT cells with the P2 sample and MHV-A59, and extracted intracellular RNAs at various times after infection. Northern blot analysis using a CAT gene-specific probe showed accumulation of the 3.8-kb long genomic MIGCAT RNA and the 0.8-kb long subgenomic MIGCAT RNA as early as 3 h postinfection (p.i.) (data not shown), indicating that MHV transcription was active as early as 3 h p.i.

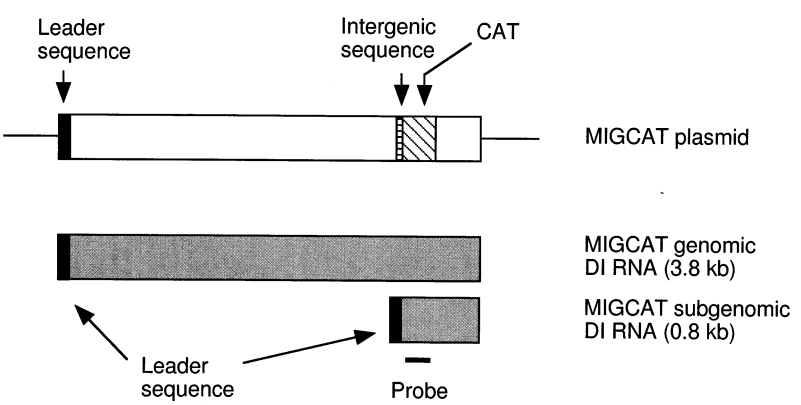

Fig. 1. Schematic diagram of the structure of MIGCAT plasmid, MIGCAT genomic DI RNA and MIGCAT subgenomic DI RNA. The location of the CAT sequence-specific probe that was used for Northern blot analysis is also shown.

For UV-irradiation of MIGCAT DI particles, 200 μl of P2 sample were spread on a 35-mm plastic dish and exposed to UV-light (wavelength, 253 nm), from 0 to 45 s, at 0°C. A 30-W germicidal lamp at a distance of 40 cm was employed for this purpose. In the experimental group, DBT cells were initially infected with helper MHV-A59, and 3 h later these cells were superinfected with the UV-irradiated P2 sample. In the control group, the DBT cells were coinfected with helper MHV-A59 and UV-irradiated P2 sample. MHV RNA replication activities, but not transcription activities, were active very early in infection (An et al., 1998) therefore, we expected that MIGCAT genomic DI RNA replication, but not transcription, would start immediately after coinfection in the control group; we expected to see similar susceptibility to UV-light irradiation of MIGCAT subgenomic and genomic DI RNAs, because subgenomic-size MIGCAT DI RNA should be synthesized after replication of genomic MIGCAT DI RNA. Intracellular RNAs were extracted 7 h p.i. of MHV, and MIGCAT-specific RNAs were detected by Northern blot analysis, using a CAT gene-specific probe (Fig. 2A,C), in which digoxigenin-11-dUTP was incorporated (DIG system, Boehringer Mannheim). After hybridization of probe to immobilized RNAs on the membrane, an anti-digoxigenin antibody–alkaline phosphate conjugate was added to the membrane, to allow binding to the hybridized probe. The RNA signal was detected on an X-ray film after treating the membrane with a chemiluminescent alkaline phosphatase substrate. Accumulation of genomic MIGCAT DI RNA and subgenomic MIGCAT DI RNA were quantitated by densitometric scanning of the autoradiograms, and susceptibility of MIGCAT-specific RNAs to UV-irradiation was plotted (Microsoft, Cricket graph) (Fig. 2B,D). Comparison of the accumulation of subgenomic MIGCAT DI RNA to genomic MIGCAT DI RNA showed that in both the experimental and control groups, the genomic and subgenomic DI RNAs had very similar susceptibilities to UV-irradiation. These data indirectly suggested that (−)-strand subgenomic MIGCAT DI RNAs that might act as templates for subgenomic MIGCAT DI RNA synthesis were not synthesized from the input single-stranded (+)-strand genomic MIGCAT DI RNA prior to synthesis of subgenomic MIGCAT DI RNA. Our data also suggested that synthesis of (−)-strand genomic-size MIGCAT DI RNA occurred prior to the synthesis of (+)-strand subgenomic MIGCAT DI RNA, indicating that synthesis of full-length (−)-strand genomic RNA is probably a prerequisite for subgenomic MHV RNA synthesis.

UV-irradiation of coronavirus-infected cells has been used to understand whether coronavirus mRNAs are produced by processing or splicing of larger precursor molecules (Jacobs et al., 1981; Stern and Sefton, 1982; Yokomori et al., 1992; Den Boon et al., 1995). In these UV-transcription mapping studies, UV-lesions are introduced into the (−)-strand template RNAs that accumulate during coronavirus replication. A study of UV-irradiation of MHV-infected cells at 2.5 or 3 h p.i. suggested that MHV mRNA synthesis requires the presence of a genomic-length RNA template early in infection (Yokomori et al., 1992). Their conclusion is consistent with our present data. UV-irradiation late in coronavirus infection showed that a UV-target size of the template for mRNAs is very similar to their physical size (Jacobs et al., 1981; Stern and Sefton, 1982; Yokomori et al., 1992; Den Boon et al., 1995), indicating that coronavirus mRNAs are not accumulated by the processing of a genomic-size (+)-strand precursor RNA. These UV-transcription mapping studies late in infection, however, did not clarify whether mRNAs are synthesized from a genomic-size (−)-strand template RNA or each mRNA is synthesized from the (−)-strand template RNA of its own size. Although UV-irradiation was used in the present study, the aim of UV-irradiation and UV-irradiation procedure were different from the previous UV-transcription mapping studies. We specifically tested the possibility that (−)-strand subgenomic RNAs are synthesized from the full-length (+)-strand genomic RNA prior to subgenomic mRNA synthesis. For this purpose, UV-lesions were introduced to (+)-strand MIGCAT DI RNA in virus particles and UV-unirradiated helper virus was provided for the replication of UV-irradiated MIGCAT DI RNA.

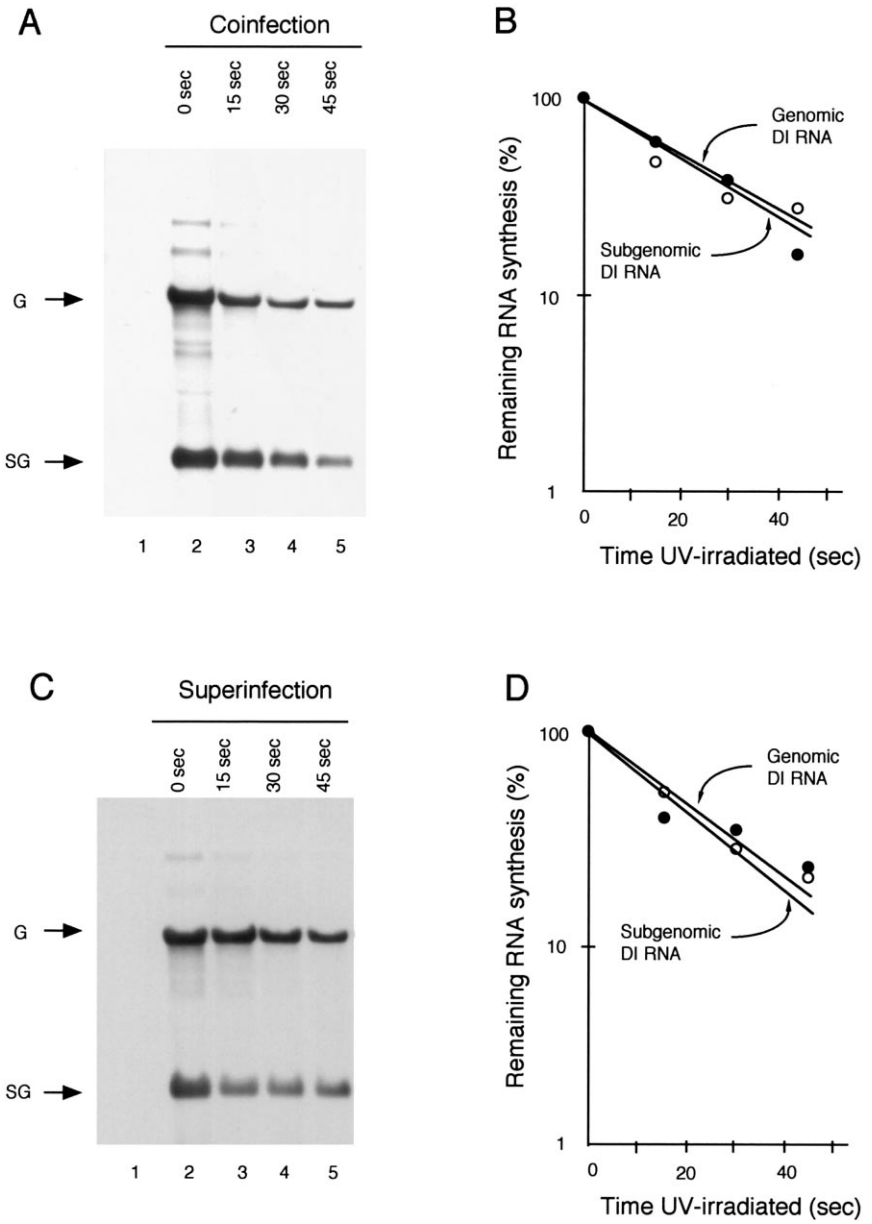

Fig. 2. Effect of UV-irradiation on accumulation of MIGCAT genomic DI RNA and MIGCAT subgenomic DI RNA. (A) The P2 virus sample was UV-irradiated for 0 s (lane 2), 15 s (lane 3), 30 s (lane 4), or 45 s (lane 5). DBT cells were infected with MHV-A59 (lane 1), or coinfected with MHV-A59 and UV-irradiated P2 samples (lanes 2–5). Intracellular RNAs were extracted at 7 h p.i., and accumulation of MIGCAT-specific DI RNAs were examined by Northern blot analysis using a CAT-specific probe. (C) MHV-infected DBT cells were superinfected with UV-irradiated P2 sample at 3 h p.i. of MHV; the length of UV-irradiation were for 0 s (lane 2), 15 s (lane 3), 30 s (lane 4), or 45 s (lane 5). DBT cells were infected with MHV-A59 alone (lane 1). Intracellular RNAs were extracted at 4 h p.i. of UV-irradiated P2 sample. Accumulation of MIGCAT-specific DI RNAs were examined by Northern blot analysis using a CAT-specific probe. (B,D) The radioactivity of the MIGCAT genomic and subgenomic DI RNAs in the gels shown in (A) and (C), respectively, were quantitated and expressed graphically. Open circle, MIGCAT genomic DI RNA; closed circle, MIGCAT subgenomic DI RNA.

Our present data, suggesting that the synthesis of full-length (−)-strand genomic RNA is a likely prerequisite for subgenomic MHV RNA synthesis, does not contradict the conclusion from previous UV-transcription mapping studies.

We assumed that UV-lesions introduced into genomic MIGCAT DI RNA mainly affect template function of genomic MIGCAT DI RNA. We could not completely eliminate the possibility that UV-lesions introduced into genomic MIGCAT DI RNA may also affect some undescribed biological functions of MIGCAT genomic RNA that work prior to initiation of (−)-strand RNA, however, this has not been implicated in previously published studies. It is possible that the introduction of UV-lesions in genomic MIGCAT DI RNA inhibits translation of MIGCAT DI-specific protein, and suppression of MIGCAT-specific protein synthesis may affect the initiation of (−)-strand RNA. This is less likely, because translation of a DI-specific protein of MHV–JHM-derived DI RNA, from which MIGCAT DI RNA was constructed, is not necessary for DI RNA synthesis (Liao and Lai, 1995). It has been postulated that sequences near the 5′-region and 3′-end of MHV genomic RNA may interact, and that this interaction is important for RNA synthesis (Lin et al., 1996). Consequently, there is a possibility that the introduction of UV-lesions may damage the structure of the 5′-region of genomic MIGCAT RNA and suppress (−)-strand RNA synthesis. However, it is reported that the very 3′-end 55 nt plus poly (A) is sufficient for MHV (−)-strand synthesis (Lin et al., 1994). Therefore, putative structural change(s) near the 5′-region of genomic MIGCAT DI RNA by UV-lesions probably did not affect (−)-strand RNA synthesis.

The most straightforward interpretation of the present data is that the (−)-strand genomic-size MIGCAT DI RNAs, which are synthesized from input genomic MIGCAT DI RNA, were used as a template for the synthesis of subgenomic MIGCAT DI RNA; subgenomic-size (+)-strand MIGCAT may be synthesized by either a leader-primed transcription mechanism, polymerase-jumping mechanism, or processing of genomic-size precursor (+)-strand MIGCAT DI RNAs. Alternatively, our data may indicate that the synthesis of (−)-strand genomic-size RNA is required for the synthesis of (−)-strand subgenomic RNA from the (+)-strand genomic MIGCAT DI RNA. Perhaps (−)-strand subgenomic RNA synthesis requires a template that is a double-stranded. Initiation and elongation of MHV (−)-strand RNA synthesis does not require a double-stranded RNA structure, because MHV (−)-strand RNA synthesis must occur from the input MHV single-stranded genomic RNA in MHV-infected cells. Accordingly, if (−)-strand subgenomic MHV RNA is synthesized from double-stranded genomic-size MHV RNA prior to subgenomic mRNA synthesis, then this possible requirement for a double-stranded RNA structure would probably center on termination of (−)-strand subgenomic RNA synthesis.

MIGCAT DI RNA was replicated and transcribed after superinfection of MIGCAT DI particles into MHV-infected cells. This indicated that there was no strong homologous interference between helper MHV and superinfecting MIGCAT DI particles. The strong homologous interference activity described in alphavirus infection (Adams and Brown, 1985) does not seem to exist in MHV infection. The present data were consistent with our previous finding that in those cells that had been pre-infected with MHV, transfection of in vitro synthesized DI RNA results in replication and transcription of MHV DI RNA (Jeong and Makino, 1992).

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