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A Subgenomic mRNA Transcript of the Coronavirus Mouse Hepatitis Virus Strain A59 Defective Interfering (DI) RNA Is Packaged When It Contains the DI Packaging Signal

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In infected cells, only the genomic RNA of the coronavirus mouse hepatitis virus strain A59 (MHV-A59) is packaged into the virions. In this study, we show that a subgenomic (sg) defective interfering (DI) RNA can be packaged into virions when it contains the DI RNA packaging signal (DI RNA-Ps). However, the sg DI RNA is packaged less efficiently than the DI genomic RNA. Thus, while specificity of packaging of RNAs into MHV-A59 virions is determined by the DI RNA-Ps, efficiency of packaging is determined by additional factors.

Coronaviruses are enveloped positive-strand RNA viruses, and during replication of the 28- to 32-kb genome, a 3′-coterminal nested set of mRNAs from which the different viral proteins are translated is produced (5, 17). Only the genomic RNA is detected in the virions of the murine coronavirus mouse hepatitis virus strain A59 (MHV-A59) (14), although trace amounts of mRNA 7 of strain JHM have been detected in purified virions (7). The specific encapsidation of MHV genomic RNA is in keeping with the identification of a defective interfering RNA packaging signal (DI RNA-Ps) in the open reading frame 1b (ORF1b) region of the replicase gene (3, 14). In MHV strain JHM, it was shown that a 69-nucleotide (nt) hairpin within the DI RNA-Ps was sufficient for packaging (3). Such a structure is also predicted in the MHV-A59 DI RNA-Ps (3). The subgenomic (sg) RNAs do not contain this encapsidation signal, and this might explain why they are not packaged into virions.

Whereas MHV-A59 packages only genomic and DI RNAs into the virions, several other coronaviruses can also package viral mRNAs. sg mRNAs are detected in virions of bovine coronavirus (BCV), transmissible gastroenteritis virus, and infectious bronchitis virus (4, 11, 12, 20). The N and M mRNAs of BCV were shown to be packaged as efficiently as the genome (4). However, the mRNAs of both transmissible gastroenteritis virus and infectious bronchitis virus were detected in the virions in amounts that were 5- to 200-fold less than the genome (11, 12, 20).

To test whether a recombinant sg mRNA of MHV-A59 carrying the DI RNA-Ps would become encapsidated into virions, we inserted the intergenic sequence of mRNA3 upstream of the packaging signal in a pMIDI-HD (1) derivative. This approach allowed us to compare packaging of the sg RNA to the parental DI RNA. We have previously shown that insertion of MHV-A59 intergenic-promoter sequences into pMIDI results in the production of sg DI RNAs; however, in these cases the promoter was introduced downstream of the DI RNA-Ps (15, 18). To be able to insert a promoter upstream of the DI RNA-Ps, pMIDI-ΔH-HD was first created by deleting the *Hin*dIII fragment of pMIDI-HD. A linker containing the sequence of the mRNA3 promoter of MHV-A59 and an AUG codon directly downstream of the promoter was designed. The AUG codon was engineered such that the sg DI RNA would have an ORF proceeding until the end of the nucleocapsid gene. This was done to have the sg DI RNA resemble a coronavirus mRNA. Complementary oligonucleotides containing these sequences were mixed in equimolar amounts in a buffer containing 50 mM $NaCl_2$, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris-HCl (pH 7.5) and were annealed by slowly decreasing the temperature from 65 to 4°C. The linker was inserted into the unique *Hin*dIII site of pMIDI-ΔH-HD, resulting in plasmid pMIDI-sg1 (Fig. 1). The orientation of the linker was verified by sequencing. The linker did not interrupt the ORF of pMIDI-ΔH-HD, which was shown to be important for the fitness of MIDI derivatives (2, 16). The integrity of the ORF was checked by in vitro translation (data not shown).

L cells were transfected with in vitro-transcribed RNA of pMIDI-sg1 or pMIDI-ΔH-HD and were subsequently infected with helper virus MHV-A59 at a multiplicity of infection (MOI) of 10 to replicate the DI RNA. Intracellular RNAs of several undiluted passages were isolated (13) and analyzed by gel hybridization (9). Analysis of intracellular RNA with a Ps-specific oligonucleotide probe (061MHV) showed the presence of genomic, DI, and subgenomic DI RNAs after passage 2 (Fig. 2). No sg DI RNA was detected in cells that contained the MIDI-ΔH RNA (Fig. 2, lane ΔH). A new DI RNA also arose, which is often observed upon undiluted passaging of helper virus. Since the DI RNA with the extra promoter could be passaged, we concluded that MIDI-sg1 RNA was both replication and packaging competent. These data did not reveal whether the sg mRNA generated from this DI RNA was packaged into virions.

To analyze encapsidation of the sg DI RNA, viral RNA was isolated from sucrose gradient-purified virus. Several undiluted passages were performed to obtain sufficient amounts of virus to be able to detect the sg DI RNA. P4 medium was harvested at 16 h postinfection and was cleared by centrifugation. Virus was precipitated with polyethylene glycol overnight; 250 μl of Trans ^{35}S-labeled (Met plus Cys; ICN) MHV-A59 was added, and the mixture was subsequently purified in sucrose gradients as described elsewhere (13). The peak fractions were pooled, and the virus was pelleted. Viral RNAs were isolated from the pelleted virus as described elsewhere (13). After separation on a denaturing gel, RNAs from purified virus and from the P4 cells were subjected to Northern blot hybridization with oligonucleotide probe 061MHV. Figure 3 (lane 1) shows that the sg

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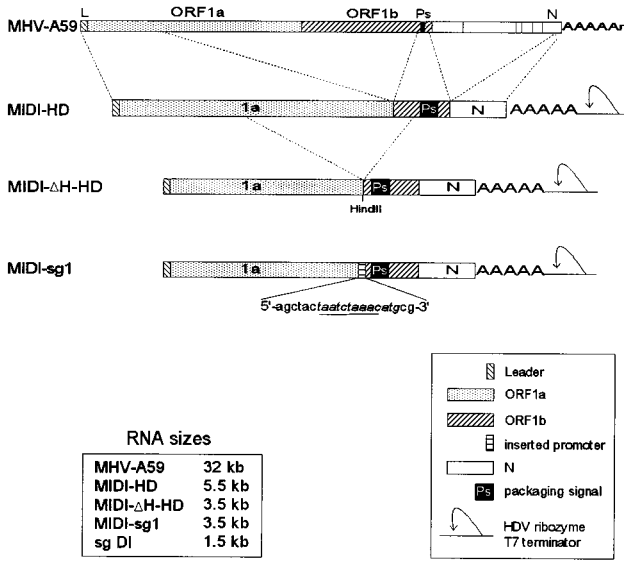

FIG. 1. Construction of pMIDI-sg1. pMIDI-HD was digested with *Hin*dIII and religated, yielding pMIDI-ΔH-HD. A linker containing the sequence of the RNA3 promoter of MHV-A59 (underlined) followed by an AUG codon was inserted into the unique *Hin*dIII site. Boxes indicate the lengths of the RNAs (left) and define the shadings (right).

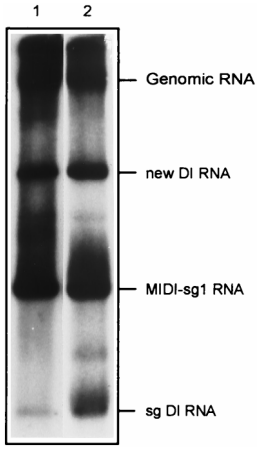

FIG. 3. Analysis of RNA from purified virus. L cells were transfected with 10 μl of in vitro-transcribed RNA of pMIDI-sg1 with the use of Lipofectin. The cells were subsequently infected with MHV-A59 (MOI of 10). After several undiluted passages, intracellular RNA was isolated from P4 cells (lane 2) and viral RNA was isolated from purified P4 virus (lane 1). RNAs were separated on agarose gels, transferred to membranes, and hybridized to oligonucleotide 061MHV. Lane 1 was exposed for a longer period than lane 2.

DI RNA was indeed packaged into virions. Rehybridization with a 3′-end probe did not reveal any additional RNAs in the virions (as shown before [14]), indicating that none of the viral mRNAs (in some cases highly abundant) that lack the RNA-Ps were packaged (data not shown).

To prove that the sg DI RNA is the sg RNA transcript from the RNA3 promoter of MIDI-sg1, reverse transcription-PCR (RT-PCR) was performed. P4 intracellular RNA from either MIDI-sg1- or MIDI-ΔH-HD-transfected cells and RNA from purified virus were subjected to RT-PCR with oligonucleotide 060MHV and the leader-specific oligonucleotide c001 (Fig. 4A). This approach predicts an sg DI RNA-specific product of 530 bp and a DI-specific product of 2.2 kb. Indeed, a PCR product of the predicted size (530 bp) was detected with both P4 intracellular and viral RNAs from MIDI-sg1-transfected cells (Fig. 4B, lanes 4 and 1). When P4 intracellular RNA from MIDI-ΔH was used as a template, the 530-bp fragment could not be detected. In all cases, the 2.2-kb fragment from the DI genomic RNA (but also a 435-bp fragment) was detected. Two extra PCR products of approximately 850 and 1,100 bp which were absent in the viral RNA were observed when intracellular RNA was analyzed (Fig. 4B, lanes 3 and 4). The 435- and 530-bp fragments were purified from the gel, cloned into plasmid PCRII (InVitrogen), and sequenced. The results showed that the 530-bp fragment consisted of the leader sequence fused to the RNA3 promoter sequence followed by the sg DI body sequence. This demonstrates that sg DI RNA found in the intracellular and virion RNA samples is indeed the subgenomic RNA transcript derived from MIDI-sg1 RNA. Sequence analysis of the cloned 435-bp PCR fragment revealed that this band was the result of a PCR artifact, i.e., aspecific binding of oligonucleotide c001 to a region at the 5′ end of ORF1b (Fig. 4A).

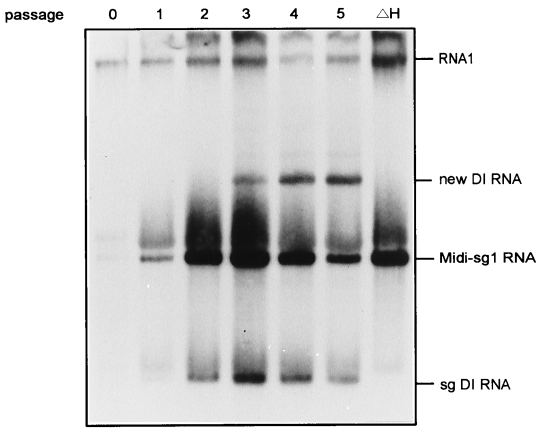

FIG. 2. Intracellular RNA analysis. L cells were transfected with 10 μl of in vitro-transcribed RNA of pMIDI-sg1 or pMIDI-ΔH-HD by the Lipofectin method. The cells were subsequently infected with MHV-A59 (MOI of 10). Several undiluted passages were performed, and intracellular RNAs from P1 to P5 cells were isolated and separated on an agarose gel. The gel was dried and hybridized with 100 ng of 5′-end-labeled oligonucleotide 061MHV, which was specific for the DI RNA-Ps. ΔH, P4 intracellular RNA.

To determine the efficiency of packaging of the sg DI RNA relative to that of the DI RNA, the RNA bands from the Northern blot shown in Fig. 3 were quantitated with a Betascope (Betagene). In the intracellular RNA, the MIDI-sg1 genomic RNA was 5.5 fold more abundant than its sg DI RNA. This difference was increased in the virion; here, the DI genome levels were 33-fold higher than those of the sg DI RNA. From the difference between the cellular and viral ratios of DI genomic and sg DI RNAs, we conclude that the DI genomic RNA was packaged into virions approximately six times more efficiently than the sg DI RNA. Whether the sg DI RNA is copackaged or packaged separately could not be established.

Our studies show that a synthetic sg RNA of MHV-A59 could be packaged specifically, when it contained the region that was previously identified as the DI RNA-Ps (3, 14). What determines the specificity of packaging? Since the mRNAs of MHV-A59 are not packaged into virions (14), the leader sequences, the N sequences, and the 3′ nontranslated region (NTR), which are common in the sg DI RNA and the viral mRNAs, do not determine specificity by themselves. The sg DI

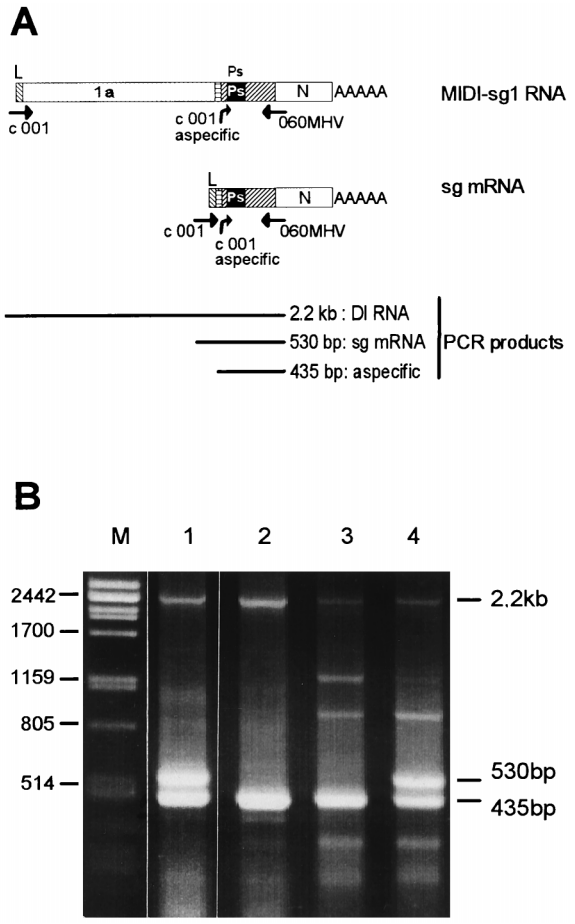

FIG. 4. (A) Schematic representation of the DI-specific RT-PCR. Binding locations of the oligonucleotides are shown. PCR products and their sizes are indicated. Shadings are as indicated in Fig. 1. The horizontally hatched box represents the inserted promoter. (B) RT-PCR analysis of P4 intracellular and viral RNA. Lanes: M, λ∗Pst molecular size marker (the sizes of several bands are indicated on the left; bp); 1, viral RNA MIDI-sg1; 2, viral RNA MIDI-ΔH; 3, intracellular RNA MIDI-ΔH; 4, intracellular RNA MIDI-sg1. Sizes of PCR products are indicated.

RNA lacks the rest of the 5′ NTR and ORF1a sequences of MIDI-ΔH. Therefore, these sequences cannot be involved in specificity either. Thus, the DI RNA-Ps present on the sg DI RNA determines specificity. During preparation of the manuscript, Woo et al. (19) published data showing that an RNA consisting of non-MHV sequences and only the 69-nt hairpin from the DI RNA-Ps of MHV strain JHM was packaged into virions. This indicates that the hairpin is sufficient for encapsidation of RNAs into MHV strain JHM virions. However, it was not established how efficiently packaging of the heterologous RNA had occurred (see below). We cannot exclude that there are strain-dependent differences in the specificity of the packaging process.

We showed that the ratio between DI RNA and the sg mRNA was different in the intracellular RNA from that in the virion RNA. This indicates that the sg RNA is not packaged as efficiently as the DI genome. Why is there a difference in packaging efficiency? Several factors between the DI genome and the sg RNA are different, such as size, presence of *cis*-acting enhancement signals, replication, and RNA structure. Efficient packaging of RNAs into MHV-A59 virions could be dependent on size. The sg DI RNA of MHV-A59 described in this paper is the smallest RNA that has ever been observed to be packaged into MHV-A59 virions. However, in the case of BCV, size is apparently not a determinant; the N and M mRNAs are detected in the virions as abundantly as the genomic RNA (4). Alternatively, the sg DI RNA could lack packaging enhancement elements and/or signals. If so, these are most likely located in the 5′ NTR downstream of the leader and/or in the ORF1a sequences of MIDI RNA. The sg DI RNA described in this paper lacks the 5′ replication signal (6) and, therefore, cannot be replicated in helper virus infected cells. Replication may play a role in packaging by, for example, helping to direct the replicating RNA to the budding compartment or the cellular compartment where the RNA is encapsidated by the nucleocapsid protein. Alternatively, replication might induce a secondary structure of the RNA that is more efficiently recognized by the nucleocapsid protein. In this view, the stem-loop structure that is located in the DI RNA-Ps (3) might be more efficiently presented upon replication of the RNA. This interaction would likely be with an RNA-binding domain that was identified in the central region of the nucleocapsid protein N (8, 10). However, such an interaction between N and the DI RNA-Ps has not yet been described. We are currently setting up an in vitro assay to be able to reveal and study this interaction.

We have recently developed a system to produce recombinant infectious DI particles in the absence of helper virus MHV-A59 (1). Thus far, only MIDI RNA was tested in this system. In order to package heterologous RNA in the virus-like particles for the development of coronavirus expression vectors, the sequences that determine packaging need to be precisely defined. The study presented in this paper shows that while specificity of packaging of RNAs into MHV-A59 virions is determined by the DI RNA-Ps, efficiency of packaging is determined by additional factors.

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