EXPRESSION OF MURINE CORONAVIRUS GENES 1 AND 7 IS SUFFICIENT FOR VIRAL RNA SYNTHESIS

Kyongmin Hwang Kim and Shinji Makino

Department of Microbiology
The University of Texas at Austin
Austin, Texas

INTRODUCTION

The genome of mouse hepatitis virus (MHV) is known to contain eight or nine genes that are encoded by mRNAs 1, 2, 2-1, 3, 4, 5 (for both 5a and 5b), 6 and 7. The gene 2, 2-1, 4 and 5a products are not essential for MHV replication at least in tissue culture¹⁻³. The 5'-most MHV gene, gene 1 most probably encodes virus RNA polymerase and proteases, the activities that are necessary for MHV RNA synthesis⁴. Gene 3 encodes S protein which is responsible for binding to cellular receptor⁵ and for induction of cell fusion⁶. MHV 5b protein is present as a virus structural protein⁷. Gene 6 encodes M protein which is believed to be essential for virus assembly. The most 3' region of the MHV genome, gene 7, encodes the N protein. N protein binds to MHV genomic RNA forming a helical nucleocapsid. Anti-N antibody inhibits MHV RNA replication in vitro⁸, indicating that N protein is necessary for MHV RNA replication. Whether the remaining proteins, S, ns5b and M are necessary for MHV RNA is not known.

An MHV-JHM defective-interfering (DI) RNA, DIssA, which is nearly genomic in size⁹, replicates by itself in the absence of helper virus infection¹⁰ and is efficiently packaged into MHV particles^{9, 11}. Almost all MHV mRNA synthesis is strongly inhibited in DIssA-replicating cells, whereas synthesis of mRNA 7 and its product N protein is not inhibited⁹. Oligonucleotide T1 fingerprinting analysis of DIssA suggested that gene 1 and gene 7 of DIssA are essentially intact, whereas multiple deletions are present from genes 2 to 6⁹.

In the present study, we examined possibilities that mRNA 7 is synthesized from DIssA template RNA, but not from helper virus template RNA, and that the gene 1 products and N protein are sufficient for the MHV RNA synthesis. Our study demonstrated that these possibilities are, in fact, the case.

MATERIALS AND METHODS

Viruses and Cells

The MHV-A59 temperature-sensitive mutant, LA 16¹², the plaque-cloned MHV-JHM, and virus sample obtained after 19 undiluted passage of original plaque-cloned MHV-JHM (JHM19th)⁹ were used. Mouse DBT cells were used for RNA transfection and propagation of viruses.

Radiolabeling of Viral RNAs and Agarose Gel Electrophoresis

Virus-specific RNAs in virus-infected cells were labeled with ³²Pi as previously described¹³ and separated by electrophoresis on 1% urea-agarose gels as described previously⁹.

Preparation of Virus-Specific Intracellular RNA and Northern Blotting

Virus-specific RNAs were extracted from virus-infected cells¹³. 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 filters¹⁴. The RNA on the filters was hybridized with ³²P-labeled probes specific for the various region of MHV RNA¹⁴.

Isolation of Clones Containing DIssA-Specific Sequence

For the amplification of a DIssA-related subgenomic RNA, cDNA was first synthesized from intracellular RNA as previously described¹⁵, using as a primer oligonucleotide 1116 (5'-CTGAAACTCTTTCCCT-3'), which binds to positive-strand MHV mRNA 7 at nucleotides 250 to 267 from the 5'-end of mRNA 7. MHV-specific cDNA was then incubated with oligonucleotide 78¹⁵, which binds to antileader sequence of MHV RNA, in PCR buffer as described previously¹⁵. DIssA subgenomic RNA-specific reverse transcriptase (RT)-PCR products were examined by Southern blot analysis in which RT-PCR products were separated by agarose gel electrophoresis and hybridized with a probe which corresponds to 1.5 to 1.7 kb from the 3'-end of MHV genomic RNA. The identified 1.2 kb-long DIssA subgenomic RNA-specific RT-PCR product was eluted from the preparative gel and cloned into TA cloning vector (Invitrogen). Clones containing DIssA-specific sequence were isolated by colony hybridization using the same probe which was used for Southern blot analysis.

RESULTS

Strategy for Analysis of DIssA-Related RNAs

We wanted to determine whether mRNA 7 detected in DIssA RNA-replicating cells was derived from the DIssA template or from helper virus genomic template. We established experimental conditions so that DIssA-derived RNA, but not helper virus-derived RNA, was efficiently synthesized. Two parental viruses were used: JHM19th and LA16, which is an MHV-A59 temperature sensitive mutant with an RNA⁻ phenotype. In JHM19th-infected cells DIssA, mRNA 7 and the 2.2 kb-long DIssE⁹ are synthesized, whereas synthesis of the helper virus-derived mRNAs is strongly inhibited at nonpermissive temperature⁹. DBT cells were infected with either JHM19th or LA16 alone, or were coinfecting with both virus samples and cultured at 32.5°C, which is the permissive temperature for LA16. After overnight

incubation, culture fluid was collected and virus samples were named passage 0 (P0) samples and these virus samples were further passaged on DBT cells several times at the permissive temperature. Because MHV-A59 usually grows about 10 times better than MHV-JHM, we expected that LA16 would grow better than MHV-JHM in coinfecting cells and that during passage, MHV-JHM would be eliminated from the virus samples.

We examined MHV-specific intracellular RNA species of these passaged virus sample at the nonpermissive temperature. Infected cells were cultured at 39.5°C or 32.5°C, and virus-specific RNAs were labeled with ^{32}P i in the presence of actinomycin D. Extracted intracellular virus-specific RNA was electrophoresed on a 1% urea-agarose gel (Fig. 1). Synthesis of DIssA and mRNA 7 was clearly apparent in the cells infected with the P3 sample from coinfecting cells, whereas synthesis of helper virus mRNAs and DIssE RNA was not detected (Fig. 1, lane 2). In addition to DIssA and mRNA 7, other minor MHV-specific RNA species were also detected (Fig. 1, lane 2 asterisks); these bands were most likely newly generated DI RNAs. Helper virus-derived mRNAs, 1 through 6, were not evident at the nonpermissive temperature in the cells infected with the P3 sample from JHM19th and LA 16-coinfected cells, therefore, the mRNA 7 shown in Fig. 1 lane 2 was most likely derived from the DIssA template RNA.

We studied the structure of the mRNA 7 by RNase T₁ 1-D oligonucleotide fingerprinting. The mRNA 7 fingerprint patterns from P1 and P3 samples of co-infected cells were very similar to that of JHM, but not to LA16 (data not shown). This data clearly demonstrated that mRNA 7 was indeed synthesized from the MHV-JHM-derived DIssA template RNA.

Primary Structure of DIssA

In addition to mRNA 7 subgenomic RNA, DIssA may synthesize small amounts of other DIssA-specific subgenomic RNAs, and these putative DIssA-specific RNAs

might be amplified by RT-PCR. Sequence analysis of cloned RT-PCR products of putative DIssA-specific subgenomic RNAs should reveal the primary structure of part of DIssA. MHV-specific cDNA was synthesized using oligonucleotide 1116. This oligonucleotide should hybridize with all MHV mRNAs and DIssA. The RT-PCR products were synthesized by incubating the cDNA with oligonucleotide 1116 and oligonucleotide 78. RT-PCR products were then examined by Southern blot analysis with a probe that specifically hybridizes to the 5'-region of gene 7. We detected a 1.2 kb-long RT-PCR product in JHM19th-infected cells, whereas we did not find this PCR product in all other samples, indicating that this RT-PCR product was synthesized from a DIssA-specific subgenomic RNA. We isolated this RT-PCR product and cloned it into a plasmid vector. We identified several clones by colony hybridization, and completely sequenced one 1.2 kb-long clone, TA23 clone.

Sequence analysis revealed that structure of TA23 was similar to MHV-JHM mRNA 2-1, but that it contained a large internal deletion spanning from nucleotide 876 in the transcription initiation site of gene 2-1 (or HE gene) to the 3'-region of gene 6. The leader-body fusion site of TA23 was the same as MHV-JHM mRNA 2-1². This sequence analysis suggested that TA23 represented a cloned cDNA of a DIssA-specific subgenomic RNA. Because coronavirus mRNA forms a 3'-coterminally nested structure, the structure of TA23 indicated that DIssA lacks all of genes 3, 4, 5, most of gene 6 and the 3'-one third of the HE gene.

We further confirmed the structure of DIssA by Northern blot analysis by using a probe corresponding to 362 - 670 nucleotides from the 5'-transcription initiation site of the HE gene. With this probe, DIssA and three minor other RNAs, approximately 5.4 kb, 3.4 kb and 2.6 kb in length, were detected in the DIssA-replicating cells (data not shown). According to the size of RNAs, these three additional RNAs most probably represented the DIssA-specific subgenomic RNAs. The size of TA23 indicated that it most likely derived from the smallest 2.6 kb RNA. Based on the sizes of the other two RNAs, the 3.4 kb RNA probably had the same structure as MHV-JHM mRNA 2, which includes a large internal deletion like that of TA23; probably the largest 5.4 kb RNA, with the same deletion, started about 2 kb from the 3'-region of DIssA gene 1. This Northern blot analysis demonstrated that DIssA synthesized three minor RNA species, in addition to mRNA 7.

Gene Products from DIssA-related RNAs Supported Replication and Transcription of Another DI RNA

Our data indicated that the gene 1 products, ns2 protein, part of the HE protein, and the N protein were expressed in the DIssA-replicating cells and that their expression was sufficient for replication and transcription of these DIssA-related RNAs. Would these gene products support replication and transcription of another DI RNA? We next examined whether these gene products supported RNA synthesis of other MHV RNA molecules in trans. MHV DIssF-derived MHV DI cDNA clone, MT 1/24 was used to examine this possibility. MT 1/24 DI RNA contains an inserted intergenic region preceding gene 7 and a subgenomic DI RNA is synthesized in MT 1/24-replicating, MHV-infected cells¹⁶. MT1/24 DI RNA was transfected into monolayers of DBT cells infected with LA16 1 hr prior to transfection¹⁶. After incubation of virus-infected cells at 32.5°C for 16 h, P0 virus sample was obtained and further passaged to generate P1 virus sample. DBT cells were coinfecting with this P1 virus sample and DIssA containing LA16 virus sample and cultured at nonpermissive temperature. Intracellular RNA was extracted from virus-infected cells and examined by Northern blot analysis (Fig. 2). Replication of MT 1/24 genomic DI RNA and transcription of MT 1/24 subgenomic DI RNA were observed in coinfecting cells, demon-

Figure 2. Northern blot analysis of intracellular RNA species. DBT cells were coinfectd with two virus samples, P2 virus sample from LA 16 and JHM19th coinfectd cells and P1 virus sample from MT 1/24-transfected, LA 16-infected cells, and cultured at 32.5°C (lanes 1 and 5) and 39.5°C (lanes 2-4 and 6). Intracellular RNA was extracted, and 1.5 µg of cytoplasmic RNA was separated on 1% formaldehyde-agarose gels and transferred to nylon filters. MHV RNA species were detected using a probe corresponding to the 3'-end of genomic RNA. An arrowhead and an arrow represent MT 1/24 genomic DI RNA and subgenomic DI RNA, respectively. Lanes 1-4 were exposed for the same length of time. Lanes 5 and 6 are shorter exposure of lanes 1 and 2.

strating that gene 1 products and N protein supported RNA synthesis of other MHV RNA molecules in trans.

The ns2 protein and the HE protein are not necessary for MHV replication^{1, 2}. Although DIssA-specific subgenomic RNA appeared to encode the ns2 protein and part of the HE protein, the 3.4 kb and 2.6 kb RNAs are most probably not necessary for synthesis of DIssA RNA and its related RNAs or for MT 1/24 DI RNA. We concluded that expression of gene 1 products and N protein was sufficient for MHV RNA replication and transcription.

DISCUSSION

The present study and an in vitro MHV RNA replication study⁸ suggested that the N protein is necessary for MHV RNA synthesis. Coronavirus nucleocapsid is not required for initiation of coronavirus RNA synthesis, because coronavirus genomic RNA extracted from purified virions is infectious^{17, 18}. Possibly a subsequent step in coronavirus RNA synthesis, e.g., genomic RNA replication, may require a nucleocapsid template. How coronavirus N protein functions in coronavirus RNA synthesis is fundamental for the understanding of coronavirus RNA synthesis.

We found that the molar ratios of mRNA 1 to mRNA 7 in the helper virus-infected cells, and of DIssA and DIssA-derived mRNA 7 in the DIssA-replicating cells were both basically the same (Fig. 1). Also no significant difference was found between the molar ratios of MT 1/24 genomic RNA to subgenomic RNA in LA 16-infected cells and in DIssA-replicating cells (Fig. 2). These data indicated that MHV transcription efficiency was not altered by the expression of the MHV S protein, ns4 protein, ns5a protein, ns5b protein, or M protein. This situation is different from influenza virus and vesicular stomatitis virus. In these viruses M protein inhibits viral RNA transcription in vitro^{19, 20}.

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