Heterogeneity of Subgenomic mRNAs of a Mutant Mouse Hepatitis Virus Strain JHM2C

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1. INTRODUCTION

Mouse hepatitis virus (MHV), a prototype of murine coronavirus, contains a single-strand, positive-sense RNA genome of ≈32 kb in length (Lee et al., 1991; Pachuk et al. 1989). Upon viral infection into susceptible cells, the viral genomic RNA serves both as an mRNA for translation of the RNA-dependent RNA polymerase polyprotein, which is required for subsequent RNA transcription and replication and as a template for the synthesis of the negative-strand RNA (Lai and Cavanagh, 1997). The genome-length, negative-strand RNA, in turn, is used for the synthesis of the viral genome. Six to seven subgenomic mRNAs (mRNAs 2 to 7) are found in MHV-infected cells; they are co-nested at the 3'-ends. Each mRNA contains a leader sequence of approximately 70 nucleotides (nt) at the 5'-end, which is identical to the leader of the genomic RNA. Depending on MHV strains, there are two to four consensus UCUAA repeats with the last repeat being UCUAAAC, at the 3'-end of the leader. An identical or similar consensus sequence is present between each gene, termed intergenic (IG) sequence, which serves as a transcription initiation signal (promoter) for subgenomic mRNA synthesis (based on the leader-primed transcription model) or a termination signal for subgenomic negative-strand RNA synthesis (based on the discontinuous transcription on the negative-strand RNA) (Lai and Cavanagh, 1997, and ref. therein). Regardless of which transcription model coronavirus actually utilizes, the IG is the *cis*-acting sequence absolutely required for subgenomic RNA transcription; it serves as

The Nidoviruses (Coronaviruses and Arteriviruses).
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a joining point between the leader (or antileader) and the remaining body part of each subgenomic RNA. This structural feature lead to propose that coronavirus RNA transcription results from RNA-RNA base-pairing between the consensus sequences of the leader and the IG template.

Previously, we analyzed the subgenomic mRNA2-1 of a mutant MHV, JHM2c and found that mRNA2-1 is heterogeneous in the leader-body fusion site (Zhang and Lai, 1994). However, it was not known whether mRNA2-1 is unique among various mRNA species of JHM2c with respect to the leader-body fusion site. In the present study, the structures of all other subgenomic mRNA species of JHM2c were analyzed. It was found that the leader-body fusion sites in all subgenomic mRNA species were heterogeneous in JHM2c, thus supporting our previous hypothesis that the leader-body fusion during transcription does not require strict RNA base-pairing between the leader and the IG.

2. MATERIALS AND METHODS

2.1 Cells and Virus

The murine astrocytoma cell line DBT (Hirano et al., 1974) was used for virus growth and infection. The naturally occurring small plaque mutant JHM2c of MHV (Makino et al. 1984) was used throughout this study.

2.2 Reverse Transcription and Polymerase Chain Reaction (RT-PCR) and Cloning of Viral Subgenomic mRNAs

For detection of viral subgenomic mRNAs, DBT cells were infected with JHM2c at a multiplicity of infection (m.o.i.) of 5. Virus grew in the presence of actinomycin D (10μg/ml). Intracellular RNAs were isolated at 7 h postinfection (p.i.) as described previously (Zhang et al., 1994). CDNAs were synthesized by RT with an antisense primer complementary to a sequence located approximately 300-nt downstream of each IG consensus sequence. The RT reaction was carried out at 42 °C for 90 min, and the PCR was performed in a thermocycler (DNA Engine, M.J. Research Inc.) for 30 cycles. The condition for each cycle was: denaturation at 95 ^{0}C for 30 seconds, annealing at 62 ^{0}C for 1 minute, and extension at 72 ^{0}C for 1 minute. PCR products were analyzed by agarose gel electrophoresis and cloned into the pTOPO2.1 TA cloning vector (In Vitrogen).

2.3 Analysis and Sequencing of cDNA Clones

All cDNA clones in the plasmid vector were analyzed by restriction enzyme digestion and agarose gel electrophoresis. Their sequences were determined with the automatic DNA sequencer (Model Prism 377, ABI) in the core facility of the Department of Microbiology and Immunology, UAMS. Either the T7 promoter primer or M13 reverse primer was used for DNA sequencing.

3. RESULTS AND DISCUSSION

It has been shown previously that mRNA2-1 of JHM2c is heterogeneous at the leader-body fusion site. To determine whether such heterogeneity is a general phenomenon in subgenomic mRNA transcription of JHM2c, DBT cells were infected with MHV JHM2c. Intracellular viral RNAs were isolated. cDNAs were synthesized with an antisense primer to each subgenomic mRNA approximately 300-nt downstream of the consensus IG site, and were amplified with a sense primer specific to the leader. PCR fragments were directly cloned into the pTOPO2.1 TA cloning vector. Inserts were analyzed by agarose gel electrophoresis following restriction enzyme digestion with EcoRI. If each subgenomic mRNA species initiates from its respective IG consensus sequence, it would be expected that all cDNAs, which represent the 5'-end of each mRNAs, would be ≈370-nt in size. However, it was found that, in addition to the major fragment of ≈370-nt, longer or shorter inserts were identified in each subgenomic mRNA species (see Fig. 1 for an example, and further data not shown). Combined with the data on mRNA2-1 (Zhang and Lai, 1994), this result suggests that each sub-genomic mRNA species of JHM2c is heterogeneous in the initiation site.

To confirm that the difference in various mRNA species is due to the difference in transcription initiation site, DNA sequencing was performed to determine the sequence of all cDNA clones at the leader-body fusion site. Sequence results revealed that the majority of the cDNA clones contained the leader-body fusion site at the consensus IG region for each subgenomic mRNA species. Interestingly, some leader-body fusion sites occurred either upstream or downstream of the respective IG consensus sequence (Fig. 2 and data not shown), thus confirming that the observed size difference of the cDNA (see Fig. 1) represents the heterogeneous initiation sites of the subgenomic mRNA species. It is possible that some initiation sites, which are located at more than 300-nt downstream of the consensus IG, would have been overlooked, because any leader-body fusion occurring further downstream could not be amplified with the 3'-primers used in the RT-PCR

(Fig. 1). However, this procedure would not exclude the mRNAs initiated upstream of the IG. Nevertheless, it is noticeable that the majority of the leader-body fusion sites are located within a few hundred nucleotides upstream or downstream of the consensus IG sequence. This suggests that the IG region including the consensus sequence provides signal(s) for initiation. It is important to note that the leader-body fusion sites of these heterogeneous mRNAs contain little sequence homology with the templates, suggesting that, even if base-pairing between the leader and the IG consensus sequence plays an important regulatory role, it is not absolutely required for subgenomic mRNA transcription. Instead, other mechanisms are likely involved in such heterogeneous mRNA initiation, i.e. RNA secondary structure or protein-RNA and protein-protein interactions (Zhang et al., 1994; Zhang and Lai, 1994). Although the present data do not provide any direct evidence in supporting the latter potential mechanism, they are consistent with, and extend the previous finding that heterogeneous mRNA initiations is a general phenomenon for JHM2c. Further experiments are needed to determine whether mRNA heterogeneity occurs in other coronaviruses (Fisher et al., 1997).

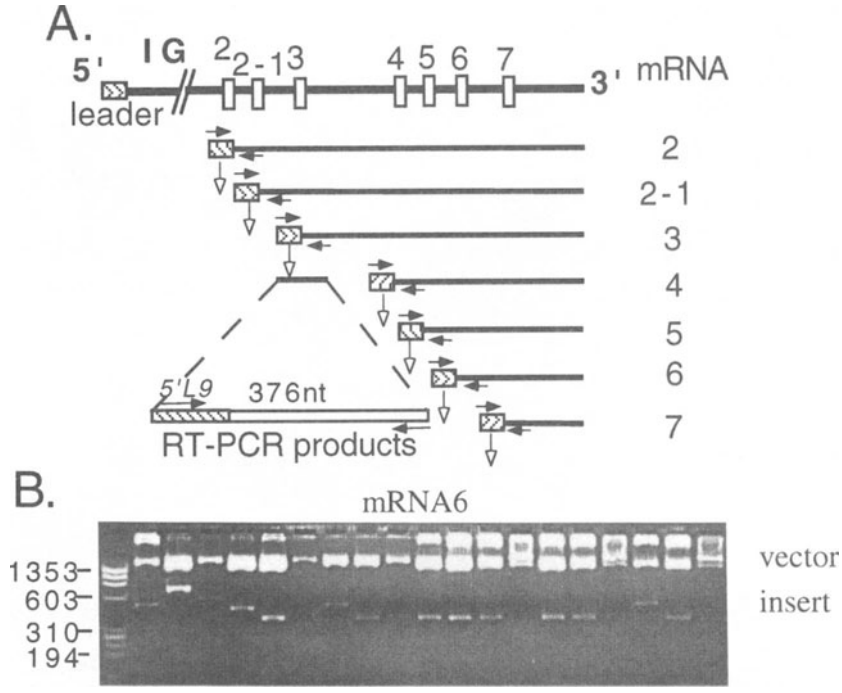

Figure 1. Strategy for RT-PCR amplification of subgenomic mRNAs of JHM2c. (A) Schematic diagram of the structure of subgenomic mRNAs and the locations of the primers. The size of the PCR fragments is indicated. (B) Heterogeneity of subgenomic mRNA 6. All clones in the pTOPO2.1 TA vector were digested with *Eco*R I, and analyzed by agarose gel electrophoresis.

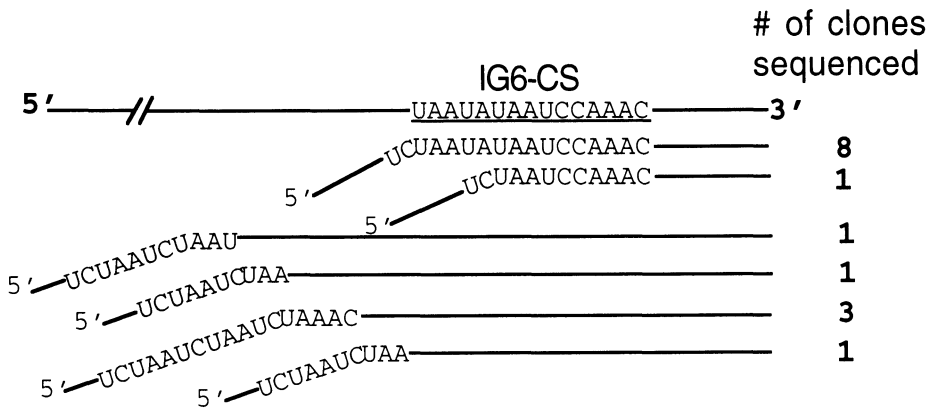

Figure 2. Summary of DNA sequencing results on the subgenomic mRNA 6. Template is shown in positive sense. Only the homology sequence between the template and mRNA is shown. Number of clones sequenced is indicated on the right.

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