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Mouse Hepatitis Virus S RNA Sequence Reveals that Nonstructural Proteins ns4 and ns5a Are Not Essential for Murine Coronavirus Replication

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Genes 4 and 5 of mouse hepatitis virus (MHV) are known to encode nonstructural proteins ns4, ns5a, and ns5b, whose function is unknown. In this study, we demonstrated that one of the MHV strains, MHV-S, did not synthesize mRNA 4 and made a smaller mRNA 5. Sequence analysis showed that the transcription initiation site for gene 4 of MHV-S was mutated from the consensus UCUAAAC to UUUAAAC, consistent with the idea that mutations in this region abolish mRNA synthesis. Furthermore, within gene 5 there were deletions totaling 307 nucleotides which deleted almost all of open reading frame 5a, but preserved open reading frame 5b of gene 5. Comparison of the growth of MHV-S with other MHV strains in DBT cells revealed no significant growth defect in MHV-S. These results suggest that ns4 and ns5a are not essential for viral replication in tissue culture cells, and thus join gene 2 and the hemagglutinin-esterase (HE) gene as nonessential viral genes in MHV.

Mouse hepatitis virus (MHV), a member of the family *Coronaviridae*, contains a single-stranded, positive-sense RNA genome of 31 kb (8, 17). The viral particle is enveloped and consists of three or four structural proteins (6). The spike (S) protein is a 180-kDa envelope glycoprotein which is frequently cleaved into two 90-kDa species and is apparently responsible for receptor binding on target cells (3, 26, 29). The membrane (M) protein is a membrane-spanning glycoprotein of 23 kDa, and the nucleocapsid (N) protein is a phosphorylated RNA-binding protein of 55 to 60 kDa (6). While these three proteins are present in every MHV isolate, a fourth structural protein, the hemagglutinin-esterase (HE) protein, is present only in certain MHV strains (23, 27). The HE protein is an envelope glycoprotein of 65 kDa whose function is unknown. These proteins are translated from virus-specific mRNAs which show a nested-set structure (7). Most MHV strains synthesize seven or eight mRNAs with common 3′ termini but unique 5′ regions (7, 11). Although these mRNAs are structurally polycistronic, only their 5′ unique regions are translated (10).

In addition to the four structural proteins, there are at least four genes that encode nonstructural (ns) proteins. The order of genes on the MHV genome is, from 5′ to 3′, ns1-ns2-HE-S-ns4-ns5a-5b-M-N (6), and the genes are encoded by mRNAs 1, 2, 2-1, 3, 4, 5 (for both 5a and 5b), 6 and 7, respectively. The gene for ns1, the biggest gene (22 kb) on the MHV genome, presumably encodes a polyprotein of 800 kDa which includes RNA polymerase, protease, and probably some additional activities (1, 8), although no distinct ns1 proteins have been detected in MHV-infected cells. ns2 protein is a 30-kDa cytoplasmic protein which, on the basis of its amino acid sequence, is postulated to have a nucleotide-binding capacity (12). ns4 protein has been detected as a 15-kDa protein in infected cells (4, 25), while the ns5 gene has two open reading frames (ORFs), 5a and 5b (2, 24). In the JHM and A59 strains of MHV, ORFs 5a and 5b have the capacity to encode a 13-kDa basic protein and a 10-kDa neutral protein with a hydrophobic N terminus, respectively (2, 9, 24). Both ORFs 5a and 5b are encoded from a single mRNA 5; the downstream ORF, 5b, was translated preferentially in vitro, suggesting the utilization of an internal AUG in this mRNA (2, 9).

Although the function of the ns proteins is not clear, two recent studies have suggested that some of these proteins are not essential for coronavirus replication. Schwarz et al. isolated a variant of the JHM strain of MHV which, despite a defective ns2 gene, had normal growth properties, suggesting that the protein was not necessary for viral replication in tissue culture (22). Furthermore, porcine respiratory coronavirus apparently lacks protein ns3a (equivalent to ns4 of MHV in genomic location) (19).

In this study, we examined a strain of MHV, MHV-S, and found that gene 4 of this virus was not expressed and gene 5a was deleted. However, the growth of MHV-S in cell culture was not significantly different from that of the other MHV strains tested. These data suggest that ns4 and ns5a are not essential for viral replication in tissue culture and add to the growing list of nonessential viral genes in the coronavirus genome.

MHV-S, which was initially isolated from mice dying of acute hepatitis (20), was a generous gift from Paul Masters of Wadsworth Center for Laboratories and Research, New York State Department of Health. A59, a prototype MHV, was used for comparison and was derived from a source described previously (28). Virus was propagated in a murine astrocytoma cell line, DBT (5), throughout the study.

We first examined the intracellular virus-specific RNA patterns of MHV-S and A59 (Fig. 1). ^{32}P-labeled RNA was extracted from infected DBT cells, denatured by glyoxal treatment, and analyzed by agarose gel electrophoresis as described previously (15). Although seven mRNAs were detected in both MHV-S- and A59-infected cells, MHV-S synthesized neither mRNA 4 nor mRNA 5. Instead, a novel mRNA (Fig. 1, arrowhead), with a size intermediate between those of mRNAs 5 and 6, was observed. Furthermore, mRNAs 1, 2, and 3 of MHV-S migrated slightly faster than the corresponding mRNAs of A59 but their relative migra-

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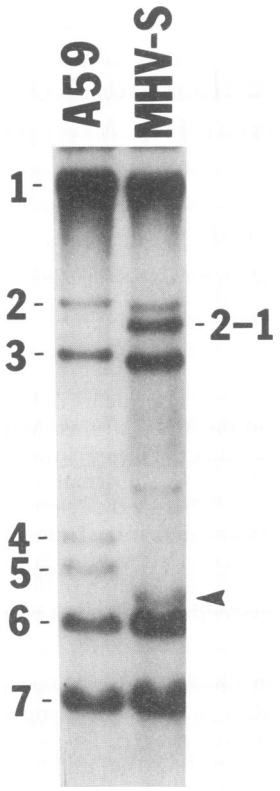

FIG. 1. Intracellular virus-specific RNA from A59- or MHV-S-infected DBT cells. DBT cells were infected with either A59 or MHV-S at a multiplicity of infection of 5 to 10. The medium was changed to phosphate-free minimum essential medium containing 1% dialyzed fetal calf serum and 2.5 μg of actinomycin D (Sigma) per ml at 3.5 h after infection. Two hours later, 0.6 mCi of $^{32}P_i$ (ICN) was added and cells were labeled for 2 h. RNA was then extracted from cytoplasm as described previously (15). The numbers indicate each viral mRNA (6). The smaller mRNA 5 in MHV-S is indicated by the arrowhead.

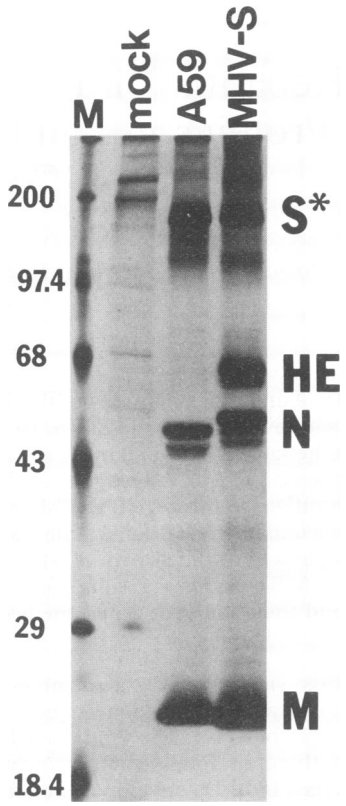

FIG. 2. Intracellular virus-specific proteins from MHV-S- and A59-infected cells. DBT cells were infected with A59 or MHV-S at a multiplicity of infection of 5 to 10 or incubated with medium only as a control (mock infection). Cells were starved for methionine for 30 min and then labeled with 50 μCi of [^{35}S]methionine (Translabel; 1,193 Ci/mmol; ICN) per ml for 20 min at 7 to 8 h after infection. Lysates were prepared as described previously (28), immunoprecipitated with polyclonal antibodies made against MHV (23, 28), and analyzed by electrophoresis in a 7.5 to 15% gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Lane M contained ^{14}C-labeled molecular weight markers (BRL), whose sizes (10^3) are indicated on the left.

tion rates remained unchanged. In contrast, mRNAs 6 and 7 of both viruses had the same migration rate. As demonstrated previously, mRNA 2-1, which encodes HE, was not detected in A59-infected cells, probably as a result of a mutation in its transcription initiation signal (12, 23, 27). The presence of several minor RNA species between mRNAs 3 and 5 was also detected. These RNAs varied with cell types and were most likely minor viral transcripts and rRNAs, since most of them were not detectable after poly(A) purification (unpublished observation). These minor RNAs have also been observed in cells infected with other strains of MHV (13, 15).

Since MHV mRNAs possess a nested-set structure (6, 7, 11), the patterns of viral mRNA detected (Fig. 1) suggested that a deletion occurred within gene 5 and possibly also in other genes upstream of gene 5 in the MHV-S genome. To identify the possible site(s) of deletion, we first examined the sizes of the intracellular viral proteins. MHV-S-infected cells were labeled with [^{35}S]methionine and immunoprecipitated with anti-MHV polyclonal antibody as described previously (27, 28). Figure 2 shows that the S and M proteins of MHV-S had the same size as those of A59. Although the N protein of A59 migrated faster than that of MHV-S, there was no difference in N gene size between these two viruses (18). These results suggested that the possible deletion was localized in the genes encoding nonstructural proteins, more likely genes 4 and 5. As predicted, the HE protein was synthesized only in MHV-S-infected cells (27).

To determine the sites of deletions within gene 4 or 5, cDNA clones corresponding to these genes were obtained after amplification by polymerase chain reaction on the total intracellular RNA of MHV-S-infected cells by using two primers: one that binds to the 5′ unique region of gene 7, no. 327 (5′-GCATTTTCTTGCCCAGGAAC-3′), and one that binds to either the leader sequence, no. 78 (5′-AGCTTTA CGTACCCTCTCTACTCTAAAACTCTTGTAGTTT-3′), or the sequence near the 3′ end of gene 3, no. 347 (5′-GG ACACCAGGACAGTATTGT-3′). The sequence used to synthesize primer 347 was obtained by direct RNA sequencing of MHV-S RNA (data not shown) by using various primers complementary to the known gene 3 sequence of JHM (21). The PCR protocol employed has been described previously (14, 16, 27). By this approach, cDNA clones covering 800 nucleotides of the 5′ unique region of mRNA 6, 1.1 kb of the 5′ unique region plus some downstream

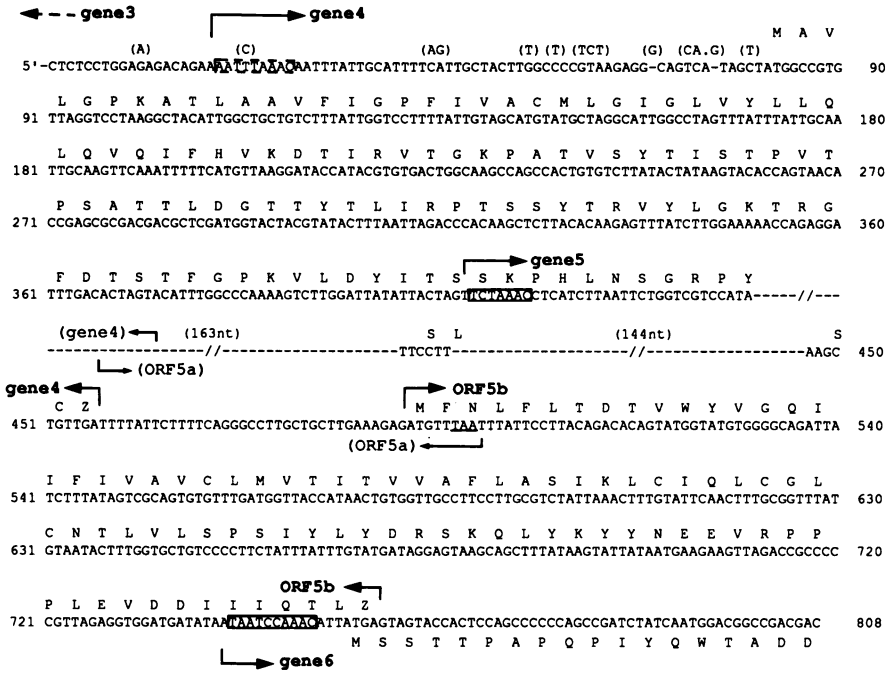

FIG. 3. Sequence of the 3'-untranslated region of gene 3, all of genes 4 and 5, and part of gene 6 of MHV-S. Boxes indicate the intergenic sites for each gene, which were determined by sequence analysis of both genomic RNA and mRNAs. The dashed box denotes the defective transcription initiation sequence of gene 4. The deleted nucleotides are represented by dashed lines, and the numbers of nucleotides deleted were determined by comparison with the published JHM sequence (24). The boundaries of genes for MHV-S are indicated. The gene and ORF designations in parentheses are the corresponding genes in JHM RNA (24).

sequences of mRNA 5, and 1.6 kb of the genomic sequence spanning genes 4, 5, and 6 were obtained and subsequently cloned into vector pTZ18U (United States Biochemical Corp.).

Following sequencing of these clones, a comparison was made with the sequences of the JHM and A59 strains of MHV (2, 24, 25). These studies revealed that while mRNA 5 of MHV-S started at the normal intergenic site for gene 5 (UCUAAAC; Fig. 3, open box), inside this gene were two deletion stretches totaling 307 nucleotides. The first deletion was 163 nucleotides long (Fig. 3) and started at a point located 42 nucleotides upstream of the first AUG of ORF 5a of JHM (24) (or 25 nucleotides upstream of the corresponding AUG of A59 (2, 24)). The second deletion was 144 nucleotides long, commencing in the middle of ORF 5a and ending 50 nucleotides upstream of the stop codon of this ORF (Fig. 3). Interestingly, between the two deletions, a six-nucleotide sequence (TTCCTT; Fig. 3), derived from the middle of ORF 5a, was retained. Thus, almost all of ORF 5a, including the initiation codon, was deleted. Since no alternative AUGs were present in the remaining sequence (other than that for ORF 5b), a truncated form of ORF 5a was unlikely to be translated. These deletions resulted in the synthesis of an mRNA 5 by MHV-S that was smaller (2.7 kb) than that of A59 (3.0 kb) (Fig. 1, arrowhead).

The sequence of MHV-S gene 4 was also determined. The transcriptional initiation site of gene 4 was mutated from AAUCUAAAC in JHM (25) to AAUUUAAAC in MHV-S (Fig. 3, dashed box). Gene 4 of MHV-S had a coding capacity of 124 amino acids which, because of the deletion described above, was shorter by 15 amino acids than the corresponding gene 4 of JHM (25), since genes 4 and 5 partially overlapped. The mutation in the transcription initiation sequence may be responsible for the failure of MHV-S to synthesize mRNA 4. We also performed a polymerase chain reaction using a leader-specific primer (no. 78) and another primer specific for the downstream region to detect any minute amount of mRNA 4 made by MHV-S; no polymerase chain reaction product of the predicted size was detected, while this product was detected in JHM-infected cells (data not shown). Thus, mRNA 4 synthesis was completely inhibited in MHV-S. In this context, we have noted previously that UUUAAAC was also present at a site upstream of ORF 1b and failed to serve as a transcription initiation sequence (8). Thus, the minor nucleotide change in this sequence could have abolished transcription of mRNA 4. It is possible that sequences neighboring UUUAAAC also contributed to the inhibition of transcription, since there was an additional base substitution at the 5' side of this transcriptional initiation sequence compared with the JHM sequence (Fig. 3). A similar example of the importance of intergenic sequences in transcriptional initiation has been demonstrated in mRNA 2-1 synthesis (23, 27). In A59, the intergenic sequence preceding gene 2-1 is UAAGCUU, in contrast to the consensus UAAACUU; correspondingly, no mRNA 2-1 was synthesized (Fig. 1). MHV-S has the consensus transcription initiation sequence for mRNA 2-1 (27), and this mRNA was synthesized (Fig. 1).

This study showed that MHV-S did not synthesize mRNA 4, probably as a result of the mutation in the transcriptional initiation sequence, and did not make ORF 5a protein because of the deletion. These defects in genes 4 and 5 did not, however, appear to affect the growth properties of MHV-S, which were similar to those of other MHV strains tested in tissue culture. For instance, the cytopathic effects of all of these viruses reached 100% at roughly the same time

in DBT cells (data not shown). Furthermore, the total amounts and peaks of synthesis of viral RNA and proteins and virus yields were similar between MHV-S and most of the other MHV strains (Fig. 1 and 2). These results suggest that ns4 and ns5a are not essential for MHV replication, at least in DBT cell culture. Different MHV strains did show minor differences in their growth properties, but none of these differences could be correlated with the presence or absence of ns gene expression.

These results add to the growing list of nonessential genes in the MHV genome. So far, ns2 (22), ns4, ns5a, and a structural protein, HE (12, 27), have been demonstrated not to be essential for MHV replication. However, these proteins may contribute to the biology or pathogenicity of the virus under certain situations. Elucidation of these functions will need additional studies.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the EMBL-GenBank data base under accession no. M64835.

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