Arch Virol (1993) 129: 301–309

Archives of Virology
© Springer-Verlag 1993
Printed in Austria

The ns 4 gene of mouse hepatitis virus (MHV), strain A 59 contains two ORFs and thus differs from ns 4 of the JHM and S strains

Brief Report

Susan R. Weiss[1], **P. W. Zoltick**[1,*], and **J. L. Leibowitz**[2]

[1] Department of Microbiology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania

[2] Departments of Pathology and Laboratory Medicine and of Microbiology, University of Texas Health Sciences Center, Houston, Texas, U.S.A.

Accepted July 29, 1992

Summary. The sequence of the MHV-A 59 non-structural gene 4 (ns 4) reveals two open reading frames. The upstream ORF potentially encodes a 19 amino acid (2.2 kDa) polypeptide and the downstream ORF potentially encodes a 106 amino acid (11.7 kDa) polypeptide. This is in contrast to MHV-JHM gene 4 which expresses a 15 kDa protein. Cell free translation of a synthetic mRNA containing both ORFs of MHV-A 59 ns 4 results in the synthesis of a 2.2 kDa poylpeptide; the predicted 11.7 kDa product of the MHV-A 59 downstream ORF is not detected during cell free translation nor in infected cells. These results add to the recent data suggesting that expression of some of the ns proteins of MHV is not necessary for efficient growth in tissue culture.

*

The murine coronavirus MHV contains a single stranded positive sense genome of approximately 31 kb [3, 10, 14]. MHV synthesizes a nested set of subgenomic mRNAs; in general, each mRNA is translated from its 5′ ORF although in at least one case a downstream ORF is also translated [4, 12]. The genome of MHV strain A 59 encodes the following structural proteins: N, the 50 kDa basic nucleocapsid protein; S, the glycoprotein that forms the spikes on the viral envelope and is responsible for attachment and induction of cell fusion and M, the transmembrane glycoprotein [18]. In addition, the genome contains an

* Present address: Department of Neurology, Thomas Jefferson University, Philadelphia, Pennsylvania, U.S.A.

approximately 22 kb gene encoding the putative viral polymerase(s) and several genes encoding small non-structural proteins. The polypeptides predicted to be encoded by these non-structural genes include the basic 30 kDa polypeptide encoded by ORF 2a [2, 22], the basic 13.9 and hydrophobic 9.6 kDa polypeptides encoded by ORFs 5a and 5b respectively [4]. Both the 30 kDa and 9.6 kDa polypeptides have been detected in infected cells using specific antisera [2, 12, 22]. The 13.9 kDa polypeptide is synthesized poorly during in vitro translation of a synthetic RNA containing ORF 5a and it is not clear whether the 13.9 kDa polypeptide is in fact synthesized during infection [4]. While it is unclear what the functions of these ns proteins are, sequence analyses suggest that the 30 kDa and 13.9 kDa polypeptides may play a role in RNA binding and the 9.6 kDa polypeptide may interact with membranes. Another non-structural polypeptide, 15 kDa in size, has been shown to be encoded by ORF 4 of MHV-JHM and has been detected, using an antibody, during infection with JHM [6]. While no function has been attributed to this 15 kDa JHM ns 4 gene product, it is predicted to be an integral membrane protein [17]. A 14 kDa protein was reported in A 59 infected cells [13, 15]; however, there are no data to link this protein to the ns 4 gene. We report here the sequence of ns 4 of MHV-A 59. We have found that the sequence of A 59 ns 4 differs from that of JHM such that deletions within the 15 kDa polypeptide coding region result in the presence of two ORFs instead of the one encoding the 15 kDa polypeptide.

Figure 1 shows the sequence of the MHV-A 59 ns 4 gene including the intergenic consensus sequences preceeding the ns 4 and ns 5 genes. This was obtained by sequencing three A 59 cDNA clones. Clone 344, obtained by cloning of oligo (dT) primed cDNA transcribed from A 59 genome RNA [5], was cleaved with Hae III and Hpa II and subcloned into the RF of M 13 bacteriophage. Subclones were sequenced using dideoxynucleotide sequencing technology and an M 13 universal sequencing primer [5]. Clones 613 and 332 were obtained from a cDNA library constructed from randomly primed cDNAs transcribed from A 59 genome RNA [14]; the ns 4 region was sequenced using oligonucleotide primers on double stranded plasmid DNAs. The sequence up to nt 110 of A 59 was confirmed by sequencing of genome RNA using an oligonucleotide primer complementary to nucleotides 123–150. This A 59 ns 4 sequence is similar to that of JHM but has single nucleotide deletions that result in frame shifts. This creates two ORFs in the A 59 sequence as opposed to one for the JHM sequence. The one base deletion just downstream of the AUG initiation codon of ORF 4 of JHM (nt 76 of the A 59 sequence) interrupts the 15 kDa protein ORF and instead results in two ORFs in this region, one 19 amino acids or 2.2 kDa and the other 106 amino acids or 11.7 kDa. (See Fig. 1 for nucleotide sequence and Fig. 2 for predicted amino acid sequence.) There is another one base deletion near the 3′ end of JHM ORF 4 (nt 389 of the A 59 sequence) that results in a protein truncated at the carboxyterminus relative to the JHM protein. We are confident that this sequence (including the deletions) is not due to cloning artifacts as it was obtained from at least two cDNA clones

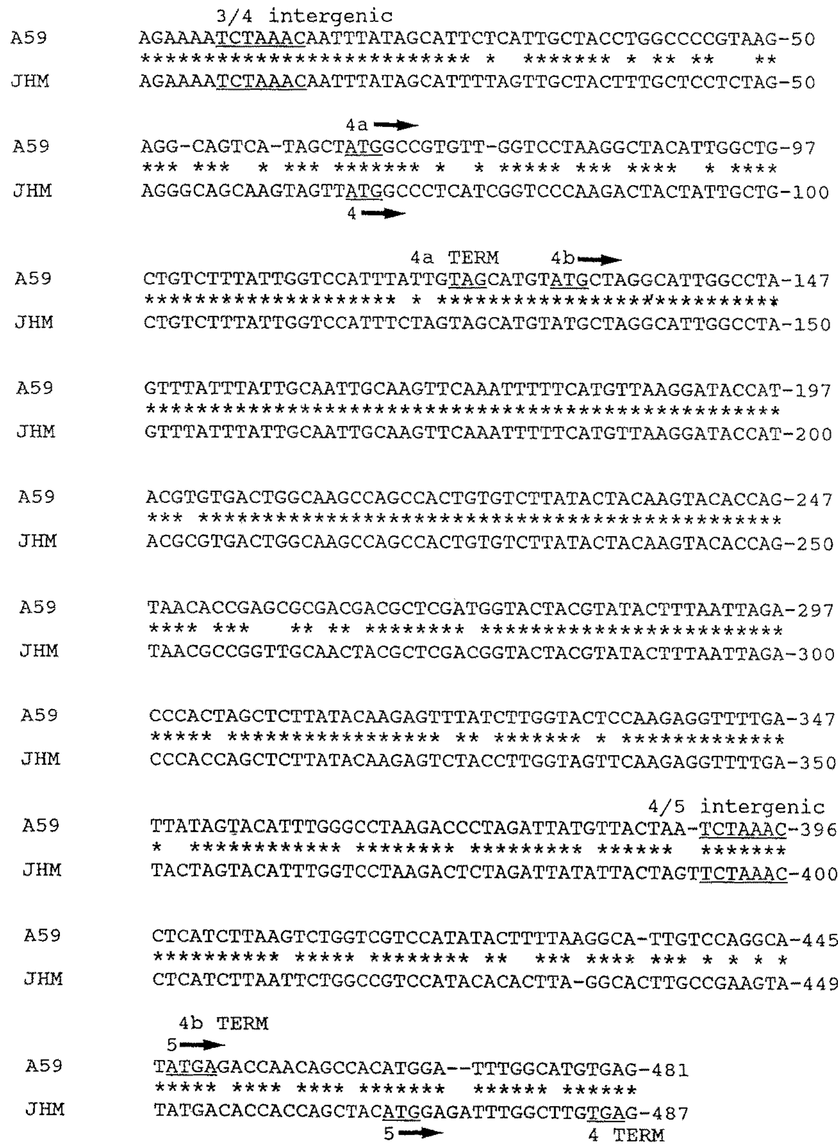

Fig. 1. Comparison of the nucleotide sequences of ns 4 of A 59 and JHM. The nucleotide sequence of the ns 4 gene of A 59 was obtained from cDNA clones 344, 613, 332. The sequence of JHM was taken from Skinner and Siddell [17] and confirmed by sequencing of pIBI 31.JHMD (described in text). The initiation and termination codons of ORFs 4, 4 a and 4 b, as well as the intergenic consensus sequences are indicated by underlining

across the entire region. Furthermore, the same sequence (up to nucleotide 110 of the A 59 sequence) was obtained from genome RNA. The viral RNA used for cDNA cloning or for RNA sequencing was derived from virus that was never more than three passages away from clonal isolation by plaque purification. Thus it is unlikely that deletions have arisen through multiple passages of virus.

```
ORF 4a                ORF 4b                         55
MAVLVLRLHWLLSLLVHLL---MLGIGLVYLLQLQVQIFHVKDTIRVTGKPATVS  A59
                      *********************************
MALIGPKTTIAAVFIGPFLVACMLGIGLVYLLQLQVQIFHVKDTIRVTGKPATVS  JHM
ORF 4

                                                    110
YTTSTPVTPSATTLDGTTYTLIRPTSSYTRVYLGTPRGFDYSTFGPKTLDYVTNL  A59
********* ************************  **** ********** *
YTTSTPVTPVATTLDGTTYTLIRPTSSYTRVYLGSSRGFDTSTFGPKTLDYITSS  JHM


                          139
NLILSLVVHILLRHCPGI-----------  A59
   *       *** *
KPHLNSGRPYTLRHLPKYMTPPATWRFGL  JHM
```

Fig. 2. Comparison of the predicted proteins encoded in ns 4 of A 59 and JHM. The nucleotide sequences of ns 4 were translated into protein sequences. The predicted proteins of A 59 ORFs 4 a and 4 b and JHM ORF 4 were aligned using the ALIGN program of PC/gene

The predicted 2.2 kDa protein encoded in ORF 4 a of A 59 would be quite hydrophobic (Fig. 3) and thus if expressed in vivo may contain a membrane spanning region. The predicted 11.7 kDa protein encoded in ORF 4 b is, for most of its length, similar to the JHM 15 kDa protein (Figs. 2 and 3). Both the A 59 and JHM proteins are predicted to be integral membrane proteins; however, because the A 59 protein is truncated at both ends there are some differences. The A 59 protein has two predicted membrane spanning domains (amino acids 1–17; 84–100) while the JHM has one (amino acid 10–26).

Like MHV-A 59, the antigenically related bovine coronavirus (BCV) genome contains two ORFs (potentially encoding 4.9 and 4.8 kDa polypeptides). Abrahams et al. [1] suggested that these ORFs could have arisen from a one-nucleotide deletion in an ORF similar to ns 4 of JHM or the 4 b polypeptide encoded by A 59 described here.

Thus, ns 4 of A 59 resembles in structure the ns 5 gene in which two ORFs follow one intergenic consensus region and are present on one mRNA. In the case of ns 5 the downstream ORF is the more frequently expressed by cell free translation and also has been the only one detected in infected cells [12]. Thus we wanted to determine which is the more likely to be translated with ns 4. The region encoding ns 4 (containing ORF 4 a and ORF 4 b) was excised from A 59 cDNA clone 613 and ligated into pIBI 31 downstream of the T 7 bacteriophage RNA polymerase promoter (pIBI 31.A 594 ab). Briefly pIBI 31.A594 ab was constructed as follows. Clone 613 was cleaved with Ssp I (about 60 nts upstream of the gene 3/4 intragenic sequence) and Dra I (about 150 nts downstream of the ORF 4 b terminator). This fragment was then treated with Bal 31. The resulting 650 nt fragment was ligated into Hinc II cut pIBI 31 downstream of the T 7 RNA polymerase promoter. This plasmid was cleaved with Hin dIII and Pst I, blunt-ended with T 4 DNA polymerase and religated. This last step removed a potential ATG initiation site for protein synthesis in the multiple

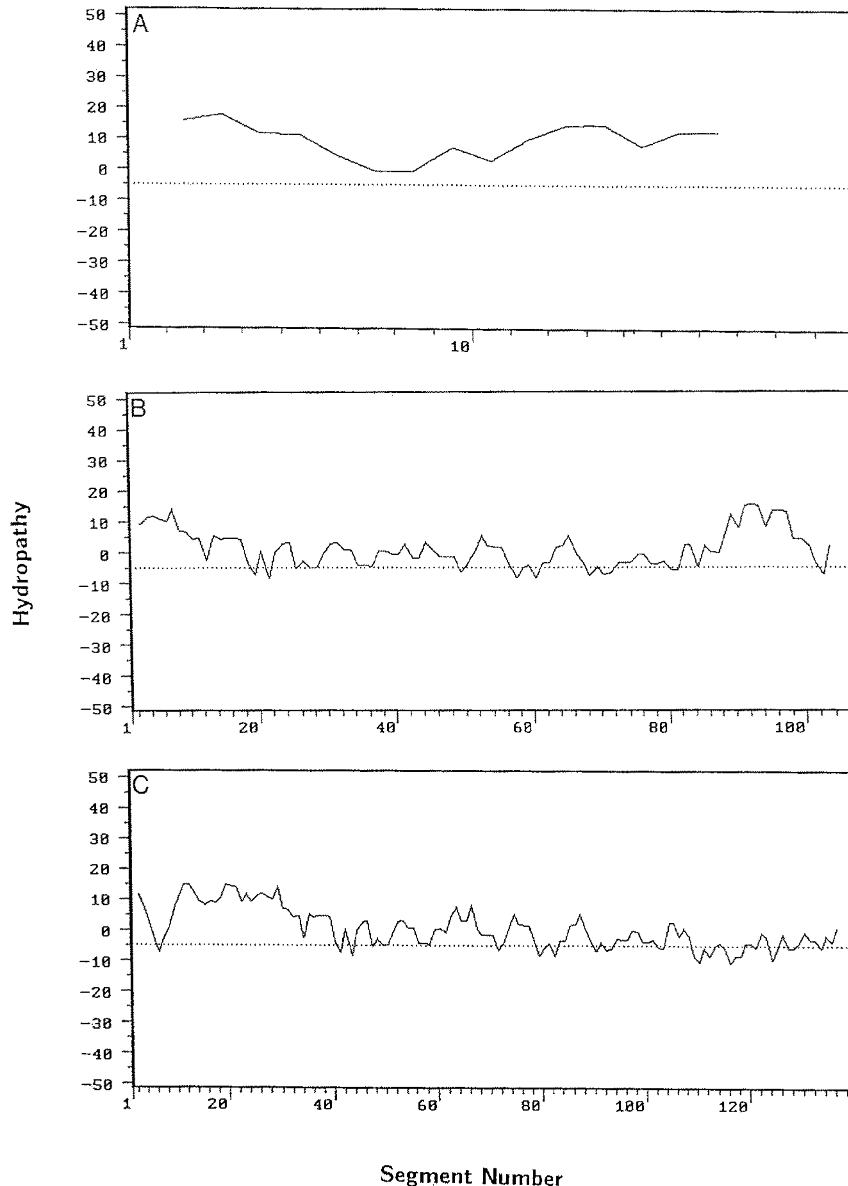

Fig. 3. Hydropathy plots for the proteins predicted from the sequences of MHV-A 59 ORFs 4 a and 4 b and JHM ORF 4. Hydropathy plots [9] were derived from the amino acid sequences shown in Fig. 2. Each bar represents the average hydropathy of a segment 5 amino acids long. Each bar is placed at the midline of the segment. The baseline (− 0.49) is the average hydropathy for most proteins that have been sequenced. **A** A 59 ORF 4 a; **B** A 59 ORF 4 b; **C** JHM ORF 4

cloning site. As a control, RNA was transcribed from a plasmid containing the ORF 4 of JHM (pIBI 31. JHM 4), encoding the 15 kDa protein. The construction of pIBI 31. JHM 4 was accomplished as follows. JHM cDNA clone 370-1 [11] DNA, spanning genes 4, 5, 6 and containing portions of genes 3 and 7 was amplified by polymerase chain reaction utilizing primers complementary to the

termini of gene 4 and cloned into the Hinc II site of pIBI 31 downstream of the T 7 promoter. The viral sequences in this clone were sequenced and the gene 4 insert found to have the identical sequence to that reported previously [17].

After linearization of plasmids pIBI 131.JHM 4 and pIBI 31.A 594 ab with Eco RI, capped RNA transcripts were synthesized using T 7 RNA polymerase in reactions containing 300 ng of template DNA as described by Krieg and Melton [8]. RNA was transcribed in vitro using T 7 RNA polymerase [8] and translated in a wheat germ lysate. The results of these translations are shown in Fig. 4. The major product of translation of the A 59 RNA is the predicted

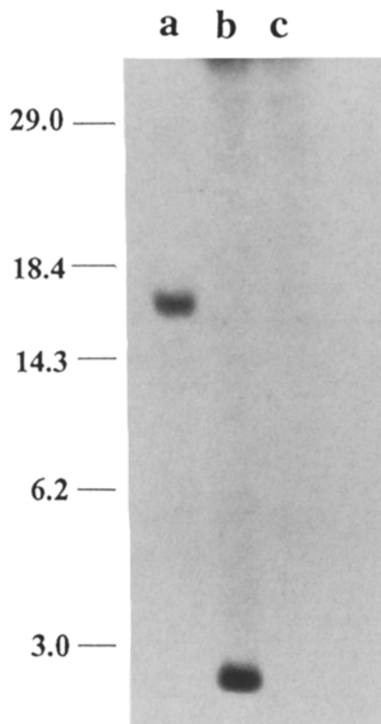

Fig. 4

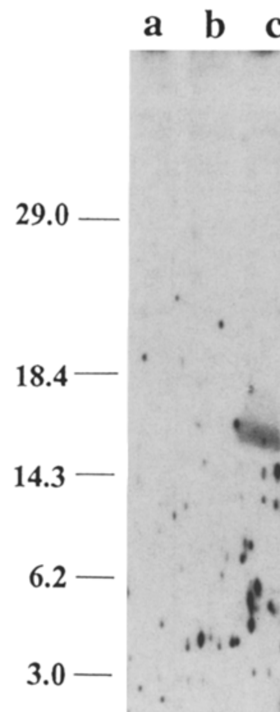

Fig. 5

Fig. 4. Cell free translation of RNAs encoding JHM and A 59 gene 4 ORFs. Approximately equal amounts of RNA, about 200 ng, were translated utilizing a wheat germ translation kit (Promega) and the translation products analyzed by SDS-PAGE in a 20% polyacrylamide gel. *a* Translation reactions programmed with transcripts representing JHM gene 4; *b* translation reactions programmed with transcripts representing A 59 gene 4; *c* translation reactions in the absence of added RNA. The positions of molecular pre-stained weight markers (BRL) are indicated to the left of the autoradiograph

Fig. 5. Detection of ORF 4 products in MHV-A 59 and MHV-JHM infected cells. Lysates of 17 Cl-1 cells, either infected with MHV-A 59 (*a*), mock infected (*b*) or infected with MHV-JHM (*c*) were electrophoresed in 15% polyacrylamide gels, transferred to nitrocellulose and analyzed by Western blot [19] using anti antiserum (1 : 200 dilution) directed against a peptide encoded in JHM ORF 4 (as described in the text). Binding of antibody was visualized using ^{125}I-labeled protein A (ICN)

2.2 kDa polypeptide encoded in the upstream ORF. Thus in vitro translation of a synthetic mRNA containing the open reading frames of ns 4 results primarily in the polypeptide encoded in the upstream ORF. This is not surprising as the AUG initiation codon for ORF 4 a is not only near the 5′ end of the RNA but also is in a better context for translation. ORF 4 a is initiated with the sequence GCU*AUG*G while ORF 4 b contains UGU*AUG*C. The consensus sequence for protein synthesis initiation by ribosome scanning is A(G)CC*AUG*GG with the − 3 and + 4 positions (the A of the AUG is position 1) being the most important for efficency of initiation of translation [7].

Given the sequence of ORF 4 a, b of MHV-A 59 and the cell-free translation results described above, it seemed unlikely that the 15 kDa protein observed in JHM infected cells would be present in A 59 infected cells. However, to confirm this, and to determine if the predicted 11.7 kDa protein was expressed from MHV-A 59 ORF 4 b, we used an antiserum directed against the JHM 15 kDa ns 4 gene product to detect products of ORF 4 in cells infected with MHV-A 59 or MHV-JHM. Since this antiserum (obtained from Dr. Stuart Siddell, Würzburg, Federal Republic of Germany) is directed against a peptide containing the 71 carboxyterminal amino acids of the JHM 15 kDa ns 4 gene product [6], it should detect an A 59 polypeptide encoded in A 59 ORF 4 b (see Fig. 2). As shown in Fig. 5, using a Western blot assay, this antiserum detected the JHM 15 kDa ORF 4 product, but did not detect a polypeptide in MHV-A 59 infected cells. Thus it is unlikely that the predicted A 59 ORF 4 b product is synthesized in infected cells. This antiserum would not be expected to detect the predicted 2.2 kDa product of MHV-A 59 ORF 4 a.

Recent data have suggested that some of the MHV small ns proteins may not be necessary for efficient replication in cell culture. MHV strains lacking expression of ORF 2 a [16] in one case and ORF 4 and 5 a [21] in another have been identified. In both these cases the corresponding mRNAs are not synthesized due to mutations in the intergenic region or deletions in the coding regions (Table 1). In the case of ns 4 of A 59, it is clear that the 15 kDa protein synthesized by JHM is not synthesized and thus is not necessary for A 59 replication in cell culture. It is also unlikely that an alternative polyprotein is expressed from

Table 1. Expression of the ns 4 gene in several strains of MHV

MHV-strain	Intergenic sequence	mRNA 4	ORFs (kDa)
JHM	AAUCUAAAC	+	15
MHV-S	AAUUUAAAC	−	15
A 59	AAUCUAAAC	+	2.2, 11.7

The data for JHM were taken from Skinner and Siddell [17] and the data for MHV-S were taken from Yokomori and Lai [21]. The proteins predicted from the ORFs were deduced from the nucleotide sequences of the ns 4 genes of the three strains. mRNA 4 of A 59 has been shown to be expressed in many publications (for example [5, 20])

MHV-A 59 ORF 4 b. However, while mRNA 4 is expressed at as least as high levels for A 59 as for JHM [20], it is not clear whether the 2.2 kDa polypeptide is indeed necessary for replication. It is possible that the differences in expression of ns 4 could influence the different pathogenic potentials of MHV-A 59 and JHM.

Acknowledgements

This research was supported by Public Health Service Grants AI-17418, NS-21954 (S.R.W.) and Multiple Sclerosis Society grant RG-2203 A-5 (J.L.L.). We thank Catherine Pachuk and Carol Budzilowicz for construction and mapping of the cDNA clones, Zofia Bielecka and Afroz Burges for technical assistance and Dr. Stuart Siddell for the antiserum directed against the JHM 15 kDa protein.

References

1. Abraham S, Kienzle TE, Lapps WE, Brian DA (1990) Sequence and expression of potential nonstructural proteins of 4.9, 4.8, 12.7 and 9.5 kilodaltons encoded between the spike and membrane protein genes of the bovine coronavirus. Virology 177: 488–495
2. Bredenbeek PJ, Noten AFH, Horzinek MC, Spaan WJM (1990) Identification and stability of a 30 kDa nonstructural protein encoded by mRNA 2 of mouse hepatitis virus in infected cells. Virology 175: 303–306
3. Bredenbeek PJ, Pachuk CJ, Noten AFH, Charite J, Luytjes W, Weiss SR, Spaan WJM (1990) The primary sequence and expression of the second open reading frame of the polymerase gene of the coronavirus MHV-A 59: a highly conserved polymerase is expressed via an efficient ribosomal frame shifting mechanism. Nucleic Acids Res 18: 1825–1832
4. Budzilowicz CJ, Weiss SR (1987) In vitro synthesis of two polypeptides from a non-structural gene of coronavirus, mouse hepatitis virus strain A 59. Virology 157: 509–515
5. Budzilowicz CJ, Wilczynski SP, Weiss SR (1985) Three intergenic regions of coronavirus mouse hepatitis virus strain A 59 contain a common nucleotide sequence that is homologous to the 3′ end of the viral mRNA leader sequence. J Virol 53: 834–840
6. Ebner D, Raabe R, Siddell SG (1992) Identification of the coronavirus MHV-JHM mRNA 4 product. J Gen Virol 69: 1041–1050
7. Kozak M (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNA. Nucleic Acids Res 12: 857–872
8. Krieg PA, Melton DA (1984) Functional messenger RNAs are producted by SP 6 in vitro transcription of cloned DNAs. Nucleic Acids Res 12: 7057–7070
9. Kyte J, Doolittle RF (1982) A simple method for displaying the hydrophobic character of a protein. J Mol Biol 157: 105–132
10. Lee HJ, Shieh CK, Gorbalenya AE, Koonin EV, LaMonica N, Tuler J, Bagdzhadzhyan A, Lai MMC (1991) The complete sequence of the murine coronavirus gene 1 encoding the putative protease and RNA polymerase. Virology 180: 567–582
11. Leibowitz JL, DeVries J (1988) Synthesis of virus-specific RNA in permeabilized murine coronavirus infected cells. Virology 166: 66–75
12. Leibowitz JL, Perlman S, Weinstock G, DeVries JR, Budzilowicz CJ, Weiss SR (1988) Detection of a murine coronavirus nonstructural protein encoded in a downstream open reading frame. Virology 164: 156–164
13. Leibowitz JL, Weiss SR, Paavola E, Bond CW (1982) Cell-free translation of murine coronavirus RNA. Virology 43: 905–913

14. Pachuk CJ, Bredenbeek PJ, Zoltick PW, Spaan WJM, Weiss SR (1989) Molecular cloning of the gene encoding the putative polymerase of mouse hepatitis virus strain A 59. Virology 171: 141–148
15. Rottier PJM, Horzinek MC, van der Zeijst BAM (1981) Viral protein synthesis in mouse hepatitis virus strain A 59 infected cells. J Virol 40: 350–357
16. Schwarz B, Routledge E, Siddell SG (1990) Murine coronavirus nonstructural protein ns 2 is not essential for virus replication in transformed cells. J Virol 64: 4784–4791
17. Skinner MA, Siddell SG (1985) Coding sequences of coronavirus MHV-JHM mRNA 4. J Gen Virol 66: 593–596
18. Spaan WJM, Cavanagh D, Horzinek MC (1988) Coronaviruses: structure and genome expression. J Gen Virol 69: 1939–1952
19. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354
20. Weiss SR, Leibowitz JL (1981) Comparison of the RNAs of murine and human coronaviruses. Adv Exp Med Biol 142: 245–259
21. Yokomori K, Lai MMC (1991) Mouse hepatitis virus S sequence reveals that non-structural proteins ns 4 and ns 5 are not essential for murine coronavirus replication. J Virol 65: 5605–5608
22. Zoltick PW, Leibowitz JL, Oleszak E, Weiss SR (1990) Mouse hepatitis virus ORF 2 a is expressed in the cytosol of infected mouse fibroblasts. Virology 174: 605–607

Authors' address: Dr. Susan Weiss, Department of Microbiology, 319 Johnson Pavilion, University of Pennsylvania, Philadelphia, PA 19104-6076, U.S.A.

Accepted July 29, 1992