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Heterogeneity of gene expression of the hemagglutinin-esterase (HE) protein of murine coronaviruses.

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Authors
Yokomori, K
Banner, LR
Lai, MM

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The hemagglutinin-esterase (HE) membrane glycoprotein is present only in some members of the coronavirus family, including some strains of mouse hepatitis virus (MHV). In the JHM strain of MHV, expression of the HE gene is variable and corresponds to the number of copies of a UCUAA pentanucleotide sequence present at the 3'-end of the leader RNA. This copy number varies among MHV strains, depending on their passage history. The JHM isolates with two copies of UCUAA in their leader RNA showed a high level of HE expression, whereas the JHM isolate with three copies had a low-level expression. In this study, the analysis of HE gene expression was extended to other MHV strains. The synthesis of HE mRNA in these viruses also correlates with the copy number of UCUAA in the leader RNA and the particular intergenic sequence preceding the HE gene. In one MHV strain, MHV-1, no detectable HE mRNA was synthesized, despite the presence of a proper transcription initiation signal. This lack of HE mRNA expression was consistent with a leader RNA containing three UCUAA copies. However, mutations and deletions within the coding region of the MHV-1 HE gene have generated a stretch of sequence which resembled the transcriptional initiation motif, and was shown to initiate the synthesis of a novel smaller mRNA. These findings strengthened the theory that interactions between leader RNA and transcriptional initiation sequences regulate MHV subgenomic mRNA transcription. Sequence analysis revealed that most MHV strains, through extensive mutations, deletions, or insertions, have lost the complete HE open reading frame, thus turning HE into a pseudogene. This high degree of variation is unusual as the other three structural proteins (spike, membrane, and nucleocapsid) are well-maintained. In contrast to bovine coronavirus, which apparently requires HE for viral replication, the HE protein in MHV may be only an accessory protein which is not necessary for viral replication. JHM and MHV-S, however, have preserved the expression of HE protein.
In addition, the mRNAs possess a common leader sequence of approximately 72 nucleotides in length, which is derived from the 5'-end of the viral genome (Spaan et al., 1983; Lai et al., 1984). The 3'-end of the leader RNA contains a pentanucleotide sequence of UCUAA, which is homologous to the intergenic sequences present at the beginning of each gene. The copy number of this pentanucleotide sequence was two to four, depending on the particular virus strain (Makino et al., 1988), and varied after serial passage of virus in cell culture (Makino and Lai, 1989a). For example, in the neurotropic JHM strain of MHV, the wild-type virus contained three copies of UCUAA; but after 10 to 12 passages, the number of copies was reduced to two (Makino and Lai, 1989a). This change was accompanied by an alteration in the pattern of mRNA expression (Makino and Lai, 1989a). Specifically, the JHM variant with two copies of UCUAA (designated as JHM(2)) made a large amount of mRNA 2-1, while wild-type JHM with three copies (JHM(3)) made only a small amount (Makino and Lai, 1989a; Shieh et al., 1989). In vitro translation studies revealed that HE protein was encoded by mRNA 2-1 (Shieh et al., 1989). Thus, HE protein expression in JHM was controlled by the copy number of UCUAA in the leader (Makino and Lai, 1989a; Shieh et al., 1989).

The HE protein possesses esterase activity similar to the receptor-destroying enzyme (RDE) of influenza C virus (Vlasak et al., 1988b), and also a hemagglutinin activity (Vlasak et al., 1988b; Parker et al., 1989). However, the hemagglutinin activity in JHM is very weak (Yokomori et al., 1989). In order to better understand the biological significance of the HE protein, we examined the structure and expression of HE in several different strains of MHV. The results demonstrated that HE expression varied at both transcriptional and translational levels amongst MHV strains. In contrast to the conserved expression of the other three structural proteins, S, M, and N, the HE gene in most MHV strains appears to be a pseudogene. The significance of the maintenance of HE expression in some of the MHV strains will be discussed.

MATERIALS AND METHODS

Viruses and cell culture

The MHV strains used in this study were from several different sources. Plaque-cloned JHM(2) and JHM(3) viruses (Makino and Lai, 1989a; Shieh et al., 1989; Yokomori et al., 1989), which have two and three copies of UCUAA in the leader sequence, respectively, and strain A59 were the same as those reported in a previous study (Yokomori et al., 1989). The two variants of JHM-DL (a large-plaque variant of JHM (Weiner, 1973; Stohlman et al., 1982)) containing two or three copies of UCUAA were obtained by a modification of the published procedures (Makino and Lai, 1989a). Briefly, JHM-DL was passaged serially without dilution in DBT cells, a murine astrocytoma cell line (Hirano et al., 1974). Virus harvested at the end of the fifth passage was passaged one additional time in DBT cells at low multiplicity of infection (m.o.i.) to eliminate possible DI particles. Virus variants were plaque-purified, and the number of UCUAA copies at the 3'-end of the leader was determined by primer extension (Makino et al., 1988). Viruses with either two or three copies of UCUAA in the leader were designated JHM DL(2) and JHM-DL(3), respectively. MHV-1 and MHV-S were obtained from Dr. Paul Masters of Wadsworth Center for Laboratories and Research, New York State Department of Health. MHV-2 has been described previously (Lai and Stohlman, 1981). Two isolates of MHV-3, i.e., MHV-3 L and MHV-3 Yac, were plaque purified from stocks originally obtained from Dr. Lucie Lamontagne of the University of Montreal. Virus was propagated in DBT cells as described previously (Makino et al., 1981b). DBT cells also were used for all the in vivo metabolic labeling experiments.

35S-labeling of intracellular proteins and preparation of cell lysates

Intracellular proteins of virus-infected DBT cells were labeled, and cell lysates made as described previously (Yokomori et al., 1989). Briefly, when the cytopathic effect (CPE) in MHV-infected DBT cells reached 95%, cells were starved in methionine-free media for 30 min, and then [35S]methionine (1193 Ci/mmol; ICN Translabel) was added to a final concentration of 50 μCi/ml. Cells were pulse-labeled for 20 min and then immediately placed on ice, washed with ice-cold phosphate-buffered saline (PBS), and lysed with lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Cell lysates were passed through a 0.2-μm syringe needle five times, transferred to Eppendorf tubes, and spun at 12,000g for 10 min at 4°C. Supernatant were harvested and stored at −70°C until required.

Immunoprecipitation and SDS–PAGE

Immunoprecipitation was performed as previously described (Yokomori et al., 1989) using rabbit hyperimmune serum prepared against purified JHM(2) virus (Yokomori et al., 1989). After immunoprecipitation, samples were denatured in 2X sample buffer (0.06 M Tris–HCl, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, and 0.1% bromphenol blue) at 37°C for 20 to 30
min, and centrifuged at 12,000g for 5 min. The supernatants were electrophoresed on 7.5 to 15% gradient or 12.5% straight polyacrylamide gels containing 0.1% SDS.

32P-labeling of intracellular RNA of virus-infected cells

DBT cells were infected with various strains of MHV at an m.o.i. of 1–5. Virus-infected cells were labeled with 200 µCi/ml of [32P]orthophosphate (ICN Biochemicals) from 5.5 to 8 hr postinfection in the presence of actinomycin D (2.5 µg/ml, Sigma). RNA extraction was performed as described below.

Preparation of virion RNA and intracellular RNA

Virion RNA was purified by a modification of the procedure described by Makino et al. (1984a). DBT cells were infected at an m.o.i. of 1–5. Culture supernatant was harvested at 15–20 hr postinfection, and cell debris removed by centrifugation at 1500 rpm for 5 min. Virus was then precipitated by the addition of ammonium sulfate to 50% saturation, and purified by ultracentrifugation twice in discontinuous sucrose gradients (Banner et al., 1990). The virus pellet was incubated at 37° for 1 hr in a solution containing 100 µg/ml of proteinase K, 100 mM Tris–HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, and 1% SDS, extracted twice with phenol/chloroform (1/1) and the RNA precipitated with ethanol. Virion RNA isolated was used for RNA sequencing and primer extension. Intracellular RNA was isolated essentially as described by Makino et al. (1984b). Briefly, infected cells were lysed at 9 hr postinfection with NTE buffer (0.1 M NaCl, 0.01 M Tris–HCl, pH 7.2, and 1 mM EDTA) containing 0.5% Nonidet P-40, and the nuclei were removed by brief centrifugation. Supernatants were treated with proteinase K and the RNA extracted as described above for virion RNA.

cDNA cloning of viral mRNAs by polymerase chain reaction (PCR)

cDNA clones corresponding to the 5′-unique coding region of mRNA 2-1 were produced using PCR as described previously (Makino et al., 1988). Intracellular RNA from virus-infected cells was annealed with primer 226 (5′-CTAACACCGCTATCCGTCAT-3′), which is complementary to the 3′-conserved region of gene 2-1, and cDNA was synthesized with reverse transcriptase (Boehringer Mannheim Biochemicals). For PCR amplification of the cDNA, a second primer, 78 (5′-AGCTTTACGTACCCTCTCTCTACTCTACAACTCTTGATGTTT-3′), which is homologous to the 5′-end of the leader plus 7 additional nucleotides containing the SnaBI restriction site, was added (Makino and Lai, 1989b). The mixture was incubated for 25 cycles of 94° for 30 sec, 54° for 90 sec, and 72° for 3 min. PCR products representing the 5′-region of mRNA 2 (2.1 kb, consisting of 0.8 kb from gene 2 and 1.3 kb from HE gene) and 2-1 (1.3 kb) were excised from low melting agarose (Sea Plaque, FMC Bioproducts, Rockland, ME) and extracted with phenol and chloroform. The cDNA was phosphorylated and blunt-ended with polynucleotide kinase and T4 DNA polymerase (Boehringer Mannheim Biochemicals), and ligated into the SmaI site of the vector pTZ18U (United States Biochemical Corp).

RNA and DNA sequencing

RNA sequencing of purified virion genomic RNA was performed using modification of the dideoxyribonucleotide chain-termination method (Sanger et al., 1977) as described previously (Banner et al., 1990). Primer 56 (5′-CGCGAATTCTGGACACGTCT-3′), which is complementary to nucleotides 172 to 188 from the 5′-end of genomic RNA (Makino and Lai, 1989b) was used to obtain the sequence of the leader RNA, including the UCUAA repeat region. For the HE gene sequence, DNA sequencing was carried out by dideoxyribonucleotide chain-termination method (Sanger et al., 1977), using primers corresponding to different regions of the gene. Some regions also were confirmed by RNA sequencing with the same primers and the method described above.

In vitro transcription and translation

Recombinant plasmid pTZ18U(HE), derived from PCR products of various strains of MHV, were constructed such that the T7 RNA polymerase promoter preceded the leader sequence and translation initiation site of the HE gene. Plasmids were linearized by digestion with XbaI and transcribed in vitro with T7 RNA polymerase as described previously (Makino and Lai, 1989b). The RNA transcripts were translated in a mRNA-dependent rabbit reticulocyte lysate or wheat germ extract (Promega Biotec) in the presence of [35S]methionine (NEN Du Pont), with or without canine pancreatic microsomal membrane (Promega Biotec). Reactions were carried out as recommended by the manufacturer.

RESULTS

Detection of intracellular virus-specific HE protein from various strains of MHV

To examine the HE expression of different MHV strains, virus infected cells were labeled with [35S]methionine, and the virus-specific proteins were im-
FIG. 1. SDS-PAGE analysis of intracellular viral proteins of different MHV strains. Virus-infected cells were radiolabeled with \[^{35}\text{S}\]methionine for 20 min at 8 to 10 hr postinfection. Cell lysates were immunoprecipitated with anti-JHM(2) rabbit serum (Yokomori et al., 1989), and analyzed by electrophoresis on 7.5 to 15% gradient polyacrylamide gels. The arrows indicate viral structural proteins. Lanes S, 1, 2, and 3 represent MHV-S, MHV-1, MHV-2, and MHV-3, respectively.

 Previously we have shown that JHM(3) synthesizes less mRNA 2-1 than JHM(2) (Makino and Lai, 1989a, Shieh et al., 1989). In this study, we confirmed this observation (Fig. 2) and extended it to include the large plaque variant of JHM, JHM-DL. Specifically, substantially more mRNA 2-1 was detected in cells infected with JHM-DL(2), which contains two copies of UCUAA, than JHM-DL(3), which contains three copies of UCUAA (Fig. 2). The amounts of HE protein synthesized by these viruses (Fig. 1) correlated well with the amounts of mRNA 2-1 synthesized.

FIG. 2. Intracellular viral RNA of different MHV strains. Intracellular RNA of virus-infected cells was labeled with \[^{32}\text{P}\]orthophosphate for 2 hr and isolated when cytopathic effect (CPE) reached 85 to 95%. RNA was denatured with glyoxal and analyzed by electrophoresis on a 1% agarose gel. Although all viral RNAs were analyzed on one gel at the same time, different lanes from the same gel were exposed for various times to adjust the intensity of the bands. Lane designations are the same as in Fig. 1. Virus-specific mRNA species are indicated by numbers, and mRNA 2-1 is indicated by small arrowheads. The novel mRNA between mRNA 2 and mRNA 3 in MHV-1 is indicated by the larger arrowhead.

mRNA 2-1 expression by different strains of MHV

To determine whether the lack of synthesis of HE protein by different MHV strains was at the transcriptional or translational level, \[^{32}\text{P}\]labeled virus-specific mRNA from virus-infected cells was examined by agarose gel electrophoresis after glyoxal denaturation. Figure 2 shows the intracellular mRNA patterns of the various strains of MHV. Surprisingly, mRNA 2-1, which encodes HE protein, was synthesized by most of the viruses, including MHV-2, and two isolates of MHV-3, even though these strains failed to synthesize any HE-related protein (Fig. 1). Thus, the mRNA 2-1 of MHV-2 and MHV-3 was nonfunctional, or defective in translational control. Consistent with their failure to express HE protein, mRNA 2-1 was not detected in A59 or MHV-1-infected cells.

Previously we have shown that JHM(3) synthesizes less mRNA 2-1 than JHM(2) (Makino and Lai, 1989a, Shieh et al., 1989). In this study, we confirmed this observation (Fig. 2) and extended it to include the large plaque variant of JHM, JHM-DL. Specifically, substantially more mRNA 2-1 was detected in cells infected with JHM-DL(2), which contains two copies of UCUAA, than JHM-DL(3), which contains three copies of UCUAA (Fig. 2). The amounts of HE protein synthesized by these viruses (Fig. 1) correlated well with the amounts of mRNA 2-1 synthesized.

Figure 2 also revealed that MHV-1, which failed to synthesize any detectable mRNA 2-1, synthesized a novel mRNA species, which was smaller than mRNA 2-1.
tein was detected in MHV-1-infected cells (Fig. 1), this and also the discrepancy between the expression of nucleotides corresponding to the leader RNA and a sequencing or DNA sequencing of PCR products. Oligonucleotide sequence analysis of HE gene

variable HE gene expression in different MHV strains from the coding region of the HE gene, the leader-5' unique mRNA also appeared to be nonfunctional.

2-1 but larger than mRNA 3. Since no HE-related protein was detected in MHV-1-infected cells (Fig. 1), this mRNA also appeared to be nonfunctional.

Sequence analysis of HE gene

We wanted to understand the molecular basis of the variable HE gene expression in different MHV strains and also the discrepancy between the expression of mRNA 2-1 and synthesis of HE protein by some viruses. Therefore, the sequences of the HE gene region of different viruses were obtained by either direct RNA sequencing or DNA sequencing of PCR products. Oligonucleotides corresponding to the leader RNA and a conserved 3'-end region of the HE gene were used as primers for PCR. Due to the nested-set structure of the coronavirus mRNAs, these clones included sequences from the coding region of the HE gene, the leader-5' unique region of mRNA 2-1, and its genomic intergenic sequences. Thus, the 5'-unique coding regions of both mRNA 2 and mRNA 2-1 were obtained. Sequence data from the HE genes of these viruses revealed substantial numbers of base substitutions, deletions, and insertions spread throughout the coding region of gene 2 in most viruses. As a result, the open reading frame (ORF) for the HE protein was truncated in A59, MHV-1, MHV-2, and both isolates of MHV-3 (Fig. 3). Specifically, in contrast to the HE genes of JHM and MHV-S, which had a coding capacity of 440 amino acids, that of A59 encoded only 14 amino acids, MHV-2, 97 amino acids, MHV-3-Y, 27 amino acids, while that of MHV-3-L was 135 amino acids. Although MHV-1 did not synthesize mRNA 2-1, its ORF was capable of encoding a protein of 268 amino acids. The usual initiating AUG of the HE gene in several viruses was also mutated or deleted (Fig. 3), thus providing the potential for additional HE gene variation in different MHV strains.

To confirm the size of the ORF predicted from the RNA sequence data, the HE genes of some of the viruses were examined by in vitro translation. Capped RNAs were transcribed in vitro from cDNA clones using T7 RNA polymerase, and then used for in vitro translation in either wheat germ extract or rabbit reticulocyte lysate system. The sizes of the 35S-labeled translation products agreed with the predicted ORF (Fig. 4A): The MHV 3 L clone yielded a 14-kDa translation product, whereas the MHV-3-Y clone yielded an 4-kDa product. Furthermore, in the proconoc of canine pancreatic microsomal membrane, MHV-3-L RNA yielded two additional protein products (Fig. 4B). The higher band possibly represented glycosylated product, since the ORF contained one potential N-linked glycosylation site. The middle band was of the same size as the primary translation product in the absence of membrane and thus represented the native product of 14 kDa. The lower band most likely represented the core protein, from which the signal peptide had been cleaved off. These results suggest that this truncated HE protein was properly translocated and processed in vitro. However, these protein products were not detected in infected cells (Fig. 1).

The UCUAA copy number in the leader and intergenic sequence of the HE gene

From studies with JHM(2) and JHM(3), it was concluded that the transcription of mRNA 2-1 depends on the interaction between the repeated UCUAA sequence motif in the leader RNA and the intergenic sequences upstream of the HE gene (Makino and Lai, 1989a; Shieh et al., 1989). Similar conclusions were reached with JHM-DL(2) and JHM-DL(3).

Sequence analysis of the intergenic regions of the HE gene and the leader RNA was further extended to all of the other viruses. Table 1 summarizes the intergenic sequences preceding the HE gene. All viruses examined contained a stretch of UA-rich sequence similar to that of JHM. The only exception was A59, which had an A to G substitution, and correspondingly, did not synthesize mRNA 2-1, despite the fact that it contained two copies of UCUAA in the leader RNA (Makino and Lai, 1989a; Shieh et al., 1989). The intergenic sequence preceding the 1IC gene was slightly different from the consensus transcription initiation motif seen in most of the other MI IV genes, i.e., gene 3 and gene 7 (Table 1). This finding may explain why gene 2-1 was regulated by the number of UCUAA copies in the leader RNA, while the other genes were constitutively expressed. Since MI IV-1 had an identical intergenic sequence preceding the HE gene (Table 1), the failure of this virus to transcribe mRNA 2-1 must have been due to the presence of three copies of UCUAA at the 3'-end of leader sequence, or some other as yet undefined reason.

The number of copies of UCUAA in the leader RNA of the various MHV strains was determined by primer extension and direct RNA sequencing of genomic RNA. The results showed that MHV-S, MHV-2, MHV-3-L, and MI IV-3-Y had two copies while MHV-1 had three (Fig. 5 and data not shown). As MHV-S, MHV-2 and both MHV-3 isolates transcribed mRNA 2-1, while MHV-1 did not (Fig. 2), these results further supported the correlation between the UCUAA copy number and expression of the HE gene. In each case, those viruses with three repeats did not transcribe mRNA 2-1 efficiently.

As described above, MHV-1 did not synthesize mRNA 2-1, but instead, made a novel mRNA smaller
and -4 have been deposited with GenBank. Their accession numbers are M-64313, M-64314, M-64315, and M-64316, respectively.

The underlined sequence beginning at the position 797 of MHV-1 indicates the intergenic site for the novel mRNA. The double-underlined sequence at the 3'-end of the gene corresponds to the primer used for PCR. The sequences for MHV-1, -2, -3, -4, 652 YOKOMORI, BANNER, AND LAI

FIG. 3. Sequence of the HE gene starting from the upstream intergenic sites. JHM and A59 sequences have been published previously (Luytjes et al., 1988; Shiie et al., 1989). Amino acid sequences are shown above the nucleotide sequence and represent that of JHM (Shiie et al., 1989). Open boxes indicate AUG initiation codons and black boxes indicate termination codons of ORFs. Dashed lines represent deletions. Insertions are shown underneath with brackets. The underlined sequence beginning at the position 797 of MHV-1 indicates the intergenic site for the novel mRNA. The double-underlined sequence at the 3'-end of the gene corresponds to the primer used for PCR. The sequences for MHV-1, -2, -3, and -4 have been deposited with GenBank. Their accession numbers are M 64313, M 64314, M 64316, and M 64318, respectively.
than mRNA 2-1 and larger than mRNA 3 (Fig. 2). By performing PCR with a pair of oligonucleotides, one identical with the 5'-end of the leader RNA and the other complementary to the 3'-end region of the HE gene, the initiation site of this novel mRNA was determined and compared with the corresponding genomic sequence (Fig. 6). As a result of mutations and a deletion in this region, the genomic sequence from nucleotides 744 to 804 (ATCCGGGTCTT) was converted to ATTTAA-TCTT, which resembled the consensus transcription initiation signal. Comparison of the genomic sequence with the sequence of the novel mRNA indicated that this was indeed the transcriptional initiation point for the mRNA species (Fig. 6). Thus, the specific expression of the novel mRNA in MHV-1 was most likely caused by the interaction of the three copies of UCUAA in the leader with the newly acquired intergenic site in the middle of the HE gene.

**DISCUSSION**

The results presented in this paper show that the functional integrity of the HE gene is not well maintained in murine coronaviruses. Of the MHV strains
Fig. 4. SDS-PAGE analysis of in vitro translation products of the HE gene of MHV-3-L (L2) and MHV-3-Y (Yac). A. RNAs transcribed in vitro from cDNA clones were translated in a wheat germ extract system in the presence of \(^{35}\text{S}\)methionine (NEN Du pont). The arrowheads indicate the translation products from MHV-3-L and two different clones of MHV-3-Y. Lane M represents \(^{13}\text{C}\)-labeled size markers (Gibco, BRL). “No RNA” lane was in vitro translation with no RNA. B. MHV-3-L RNA was translated in a rabbit reticulocyte lysate system with \(^{35}\text{S}\)methionine. The two left lanes are molecular size markers. Membrane “+” lanes represent products translated in the presence of the canine pancreatic microsomal membrane. The membrane “-” lane was translated without membrane. Products were immunoprecipitated with either monoclonal anti-HE antibody (the third lane from left) or anti-JHM(2) polyclonal antibody (the forth and fifth lanes from left, which represented different in vitro translation reactions). The specific precipitates are indicated by arrowheads: 1, glycosylated product; 2, native translation product; 3, core product from which the signal peptide had been cleaved off. The band which migrates around 30 kDa appears to be a nonspecific precipitate, since it was detected in only one out of three different immunoprecipitation reactions.

Fig. 5. RNA sequencing of the 3'-end region of the leader RNA of MHV-1 and MHV-S. Top, 5'-end; bottom, 3'-end. The brackets indicate the UCUAA pentanucleotide.

Table 1: Putative Intergenic Site: Leader Fusion Site of mRNA 2-1

| Leader | Putative Intergenic Site: Leader Fusion Site of mRNA 2-1 |
|--------|--------------------------------------------------------|
| HE gene | (UCUAA)UCUAAUCUUACUAA |
| JHM    | 5'-uaaUAAACUUuuuua |
| A59    | -uaaUAACUUuuuua |
| MHV-S  | -uaaUAAACUUuuuua |
| HNV-1  | -uaaUAAACUUuuuua |
| MHV-2  | -uaaUAAACUUuuuua |
| MHV-3L | -AaUAAAUCUAAuAa |
| MHV-3Y | -AaUAAAUCUAAuAa |
| Gene 3 (JHM) | agcaUAAUCUAAACaug |
| Gene 7 (JHM) | AAUCUAAUCUAAACUu |
| Novel mRNA | MHV-1 795 UUUAAUCUuga |
|          | (JHM 795 uCCGGuucuuga) |

Note. The leader fusion sites of mRNA 2-1 of various MHV strains were obtained from PCR products of the 5' region of the mRNA, and are compared with the corresponding regions of genomic sequences. Boldface capital letters represent common sequences between leader RNA and the intergenic sequence of the genome, and thus are likely to be the leader fusion site. Divergent nucleotides are shown in regular capital letters. Other nucleotides are shown in lower case. As a comparison, the gene 3 and gene 7 intergenic site and the novel mRNA initiation site within the HE gene of MHV-1 are shown. The sequence in parenthesis represents the corresponding JHM sequence in the HE gene.

Examined, only two viruses (JHM and MHV-S) made an intact HE protein. Other viruses had a defective gene or were defective in transcription such that no HE protein was synthesized. The failure to synthesize an HE protein was due to several different reasons in different MHV strains: (1) The mRNA for the HE protein (mRNA 2-1) was not synthesized because the number of copies of the UCUAA pentanucleotide in the leader sequence was not optimum for transcription (e.g., JHM(3) and MHV-1, which contain three UCUAA copies), or the consensus intergenic sequence (e.g., A59) was mutated. (2) The mRNA was synthesized but due to sequence alterations resulting from mutations, deletions or insertions, the usual initiation codon of the HE ORF was lost, or the reading frame closed prematurely (e.g., A59, MHV-2, and MHV-3 L and MHV-3 Y). These findings are in striking contrast to the other three MHV structural protein genes, S, M, and N, which, despite some degree of sequence diversity, are consistently expressed and the ORFs are well-maintained. These results suggest that there is no selection pressure to preserve the complete HE ORF, and consequently, this gene has drifted into a pseudogene. Furthermore, since some of the MHV strains examined in this study did not express HE, this protein is not essential for viral replication. This conclusion is consistent with the hypothesis that this gene might have been derived from influenza C virus by a fortuitous RNA-RNA recombination event (Luytjes et al., 1988). Nevertheless, the JHM and MHV-S viruses have maintained this gene despite extensive passages in tissue culture and animals.
Also, the JHM virus also with an expressed HE protein was naturally selected during JHM passages in tissue culture (Makino and Lai, 1989a). Thus, the presence of HE protein may have provided selective advantages under certain conditions. Interestingly, cold-sensitive mutants isolated from a persistent MHV-S infection of Kirsten murine sarcoma virus-transformed BALB/C cells failed to make HE protein (Yoshikura and Tejima, 1981). Also, Morris et al. (1989) have isolated JHM variants from Wistar Furth rats with a JHM-induced demyelinating disease. Besides a change in the S protein, these variants had a large deletion in the HE gene, resulting in the loss of HE protein expression (La Monica et al., 1991). These data further suggest that under certain conditions, the HE protein may provide some selection advantage.

The HE protein also is present in some other coronaviruses which have a hemagglutinin activity including BCV and human coronavirus OC43 (Vlasak et al., 1988b). The HE protein of BCV also exhibits an acetyl-esterase activity similar to the receptor destroying activity of influenza C virus (Vlasak et al., 1988b). A similar activity has been demonstrated for the HE protein of MHV (Yokomori et al., 1989). Data have been obtained which suggest that the HE protein is required for BCV infectivity. HE protein was the target of neutralizing monoclonal antibodies, which also inhibited hemagglutinin and esterase activities (Deregt and Babiuk, 1987; Parker et al., 1990). In addition, treatment with diisopropyl fluorophosphate (DFP), an inhibitor of esterase, was found to inhibit virus replication (Vlasak et al., 1988a). Similar requirements have not been demonstrated for MHV. It should be noted, however, that the presence of the HE protein does alter some biological properties of the virus. For example, in cultured cells, JHM(2) became dominant over JHM(3) after serial undiluted passages (Makino and Lai, 1989a). In mice, JHM(2) showed greater virulence and neurotropism than JHM(3) (Yokomori, unpublished data). Finally, passive immunization with monoclonal antibodies against HE protein protected mice from JHM(2) infection (Yokomori, unpublished data). These data suggested that HE may play a role in neuronal infection.

Although MHV-3 isolates examined retained a truncated HE ORF, and a protein product could be synthesized and properly processed in an in vitro translation system, no HE-related proteins could be detected in infected cells. The failure to detect these truncated HE proteins in vivo was not readily apparent. Perhaps the predicted initiating AUG was not in an ideal context in infected cells, although the sequence around this AUG agrees with the optimum translation context described by Kozak (1987). Also, the truncated protein products may degrade rapidly; however, even short pulse-labeling (15 min) did not reveal any products (Fig. 1). Additionally, because the truncated ORFs retained the putative esterase domain and signal sequence but lacked the C-terminal membrane-anchoring domain, we have investigated the possibility that the truncated HE protein may have been released into the media. No HE-specific esterase activity was detected in supernatants from infected cells (data not shown). Thus, the reason that no truncated HE protein was detected in these MHV strains remains unclear.

Another interesting characteristic of the HE gene is its transcriptional control by the copy number of UCUAA pentanucleotide sequence in the leader RNA. Both this report and previous publications (Makino and Lai, 1989a; Yokomori et al., 1989) indicate that only JHM variants with two copies of UCUAA in the leader transcribe only small amounts of this mRNA (Makino and Lai, 1989; Shieh et al., 1989). This mechanism of transcriptional control also was confirmed with a variant of JHM, JHM-DL. Furthermore, we found that other MHV strains also utilized the same transcriptional regulation. Strains MHV-S, MHV-2, and MHV-3 had two UCUAA copies and expressed a large amount of mRNA 2-1, whereas MHV-1 possessed three copies and did not express mRNA 2-1, even though it had the same intergenic sequence preceding the gene as other MHVs. In addition, MHV 1 synthesized a smaller mRNA from a novel downstream site which had a sequence similar to the...
consensus transcriptional initiation signal (UUUAUU-CUU vs UCUAUCAUCA, respectively). These results strongly suggest that MHV RNA transcription results from an interaction between the leader RNA and the intergenic sequence preceding each gene, although the precise mechanism for this transcriptional control is not yet clear. The differential control of HE gene transcription by the leader RNA may further our understanding of the mechanism of MHV transcription.

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