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In Vitro Synthesis of Two Polypeptides from a Nonstructural Gene of Coronavirus Mouse Hepatitis Virus Strain A59

CAROL J. BUDZILOWICZ AND SUSAN R. WEISS[1]

Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104/6076

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The complete nucleotide sequence of nonstructural gene 5 of coronavirus mouse hepatitis virus (MHV) strain A59 has been determined. This sequence contains two potential open reading frames which overlap by five nucleotides. The putative protein products predicted from the sequence are a basic 13,000-Da polypeptide and a 9600-Da polypeptide containing an unusually long hydrophobic amino terminus. RNAs transcribed *in vitro* from DNAs containing each of the open reading frames in pGEM vectors direct the synthesis *in vitro* of polypeptides of the sizes predicted by the sequence. An RNA transcript containing both of the open reading frames directs the synthesis primarily of the polypeptide corresponding to the downstream open reading frame. These data suggest that MHV-A59 messenger RNA 5 potentially encodes two proteins and may be preferentially translated from an internal AUG initiation codon. © 1987 Academic Press, Inc.

Coronavirus mouse hepatitis virus (MHV) strain A59 is a positive-stranded RNA virus with a genome of approximately 18 kb (Lai and Stohlman, 1978; Leibowitz *et al.*, 1981). During infection this virus generates a set of six subgenomic positive stranded RNAs as well as full-length genome RNA, all of which are transcribed from a full-genome-length negative-stranded template (Brayton *et al.*, 1982; Jacobs *et al.*, 1981). These subgenomic messenger RNAs (mRNAs) form a nested set all overlapping with genome RNA at the 3′ end (Cheley *et al.*, 1981; Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1982; Weiss and Leibowitz, 1983). Each of these RNAs contains a 5′ 70–75 nucleotide leader sequence that is probably transcribed from the 3′-end of the full-length negative-stranded RNA and may function as a primer for subgenomic RNA synthesis (Lai *et al.*, 1983; 1984; Spaan *et al.*, 1984). These RNAs are numbered 1–7 from largest to smallest.

Cell-free translation studies have demonstrated that the three MHV-A59 structural proteins, nucleocapsid (N), E1, and E2 are encoded by mRNAs 7, 6, and 3, respectively (Leibowitz *et al.*, 1982; Rottier *et al.*, 1981). These studies suggested that each virus-specific mRNA is translated from its 5′ unique region and that each unique region encodes one virus-specific polypeptide. It has been more difficult to determine coding assignments for the nonstructural proteins because they are difficult or impossible to detect with conventional virus specific antisera (Leibowitz *et al.*, 1982; Siddell *et al.*, 1982). However, cell-free translation studies have demonstrated that RNA 2 encodes a 35K (K = 1000 Da) nonstructural protein and that a pool of RNAs 4 and 5 purified from a gel directs the synthesis of a 14K nonstructural protein (Leibowitz *et al.*, 1982). More recently, sequencing studies have demonstrated that the 5′-end of mRNA 4 contains an open reading frame that potentially encodes a 15K polypeptide (Skinner and Siddell, 1985), a size similar to the product of *in vitro* translation (Leibowitz *et al.*, 1982). The products of gene 5 have been more elusive. Skinner *et al.* (1985) have reported the presence of a polypeptide of the predicted size to be a gene 5 product in infected cells; however, it was not shown to be encoded in the viral genome.

Thus, to address the question of what proteins are encoded by MHV-A59 nonstructural genes, and also to test the hypothesis that the unique region of each virus-specific RNA is utilized for the translation of one virus-specific polypeptide, we have started to clone and sequence the MHV-A59 nonstructural genes. In this communication we report the complete nucleotide sequence of gene 5 of coronavirus MHV-A59. This sequence contains two open reading frames (ORFs) potentially encoding 9.6K and 13K polypeptides. Polypeptides of these sizes have been translated *in vitro* using RNA synthesized *in vitro* from pGEM recombinant DNA templates.

MATERIALS AND METHODS

Viruses and cells

MHV-A59 (Manaker *et al.*, 1961) was grown in monolayer cultures of 17Cl-1 mouse fibroblasts. For

[1] To whom requests for reprints should be addressed.

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preparation of virus-specific RNA, cells were infected at a m.o.i. of 1 and harvested approximately 16 hr post-infection.

Preparation of virus-specific RNA

Genome RNA was purified from virions as previously described (Budzilowicz *et al.*, 1985). Intracellular RNA was extracted from the cytoplasm of MHV-A59 infected or mock-infected 17Cl-1 cells as previously described (Budzilowicz *et al.*, 1985) and poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). *In vitro* transcription of virus-specific RNA is described below.

Cloning and sequencing

Either genome RNA or poly(A)-containing RNA from virus-infected cells was used as a template and either oligo(dT)$_{12-18}$ or random oligomers of calf thymus DNA were used as primers for the synthesis of virus-specific complementary DNA (cDNA). Conversion of cDNA into double-stranded DNA and cloning into the bacterial plasmid pBR322 was carried out by conventional techniques (Gough *et al.*, 1980) as we have previously described (Budzilowicz *et al.*, 1985). Map positions of cloned fragments were determined by hybridization to Northern blots of virus specific messenger RNAs as previously described and as illustrated in Fig. 1 (Budzilowicz *et al.*, 1985). Clone g344 was derived from genome RNA by oligo(dT) priming, and clone ct54 was derived from intracellular RNA by random oligomer priming. Sequencing was accomplished by subcloning into the replicative form (RF) of bacteriophage M13, strain mp8 (Messing *et al.*, 1981) (see details in the text) and using the Sanger dideoxynucleotide chain terminating method as modified by Biggins *et al.* (1983) all as previously described (Budzilowicz *et al.*, 1985).

Construction of pGEM recombinant vectors and RNA transcription

The g344 DNA was excised from pBR322 with *Pst*I and inserted into the *Pst*I site of pGEM-1 (Promega Biotech) (see Fig. 4). The recombinant plasmid was digested with *Eco*RI, and RNA representing g344 was transcribed with SP6 polymerase. For the synthesis of RNA representing open reading frame (ORF) 1, g344 cloned DNA was digested with endonuclease *Mnl*I and the 563-nucleotide fragment containing ORF 1 was ligated into pGEM-1 (Promega Biotech) that had been digested with *Sma*I. The plasmid was digested with *Hin*dIII and RNA transcribed with T7 RNA polymerase. For the synthesis of ORF 2 RNA, g344 DNA was cleaved with *Taq*I and the resulting 1103 nucleotide *Taq*I/*Pst* fragment containing ORF 2 was ligated into the pGEM-2 that had been digested with *Pst*I and *Acc*I. The resulting plasmid was cleaved with *Hin*dIII and RNA was transcribed with SP6 RNA polymerase. Transcription reactions were carried out as described by Krieg and Melton (1984).

Cell-free translation

Approximately 1 μg of RNA synthesized *in vitro* or poly(A)-containing RNA extracted from infected or uninfected cells was translated in a wheat germ cell free lysate (Amersham). The total reaction contained 50 and 35 μl lysate and 10 μCi [^{3}H]leucine and a mixture of amino acids without leucine. Translation products were analyzed on 8–16% gradient polyacrylamide–sodium dodecyl sulfate gels (Maizel, 1971), and soaked in En^{3}Hance (New England Nuclear), dried, and fluorographed.

RESULTS

The sequence of MHV-A59 gene 5 and its putative protein products

The sequence encoded in the 5′ portion of mRNA 5, that not found in mRNAs 6 and 7, has been defined as gene 5 (Siddell *et al.*, 1982). For the purpose of sequencing this gene, cDNA clones representing this

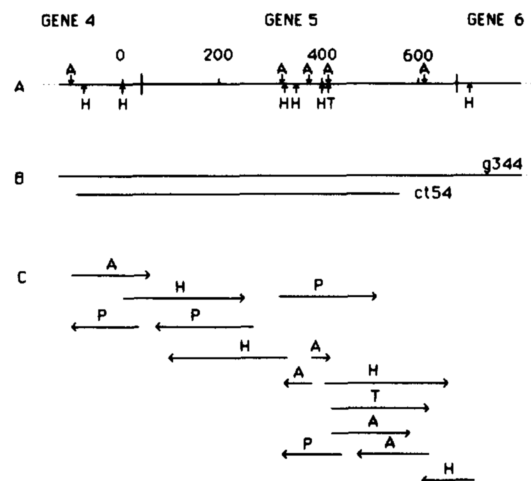

FIG. 1. Sequencing strategy for gene 5 of MHV-A59. (A) The region of the MHV-A59 genome encompassing gene 5 is shown with the relevant restriction sites, *Alu*I (A), *Hae*III (H), *Taq*I (T) marked. The slashes though the line mark the intergenic regions that define the ends of the gene as discussed in the text. (B) The locations along the genome of the two cloned viral DNA fragments g344 (1800 nucleotides) and ct54 (650 nucleotides) used for restriction digestion and subsequent subcloning into M13 RF are shown. (C) The regions and direction of sequencing of M13 subclones are shown with the arrows. The letters above the arrows designate the enzymes used (*Alu*I (A), *Hae*III (H), *Taq*I (T)) to make the subclones. Where marked P, synthetic oligonucleotide primers were used for sequencing.

portion of the viral genome were constructed and mapped on the viral genome as described under Materials and Methods. The sizes and positions of these cloned fragments as well as the strategy used for sequencing are shown in Fig. 1. The viral DNAs from these clones were digested with enconucleases *Hae*III, *Alu*I, and *Taq*I, and the resulting fragments were subcloned into the *Sma*I site of M13mp9 replicative form (Messing *et al.*, 1981) and sequenced by the dideoxynucleotide chain terminating method as modified by Biggins *et al.* (1983). Regions of the sequence that could not be determined by sequencing of M13 subclones using the universal sequencing primer were obtained by the use of synthetic oligonucleotide primer-directed sequencing on M13 DNA containing the entire g344 sequence. The DNA sequence is shown in Fig. 2.

The sequence shown in Fig. 2 includes the gene 5 coding region, the gene 4/5 and gene 5/6 intergenic regions, and the beginning of the gene 6 E1 protein coding region (Budzilowicz *et al.*, 1985). The 11-nucleotide sequence, common to at least three viral intergenic regions (Budzilowicz *et al.*, 1985) is underlined in the gene 4/5 (nucleotides 27–37) and gene 5/6 (nu-

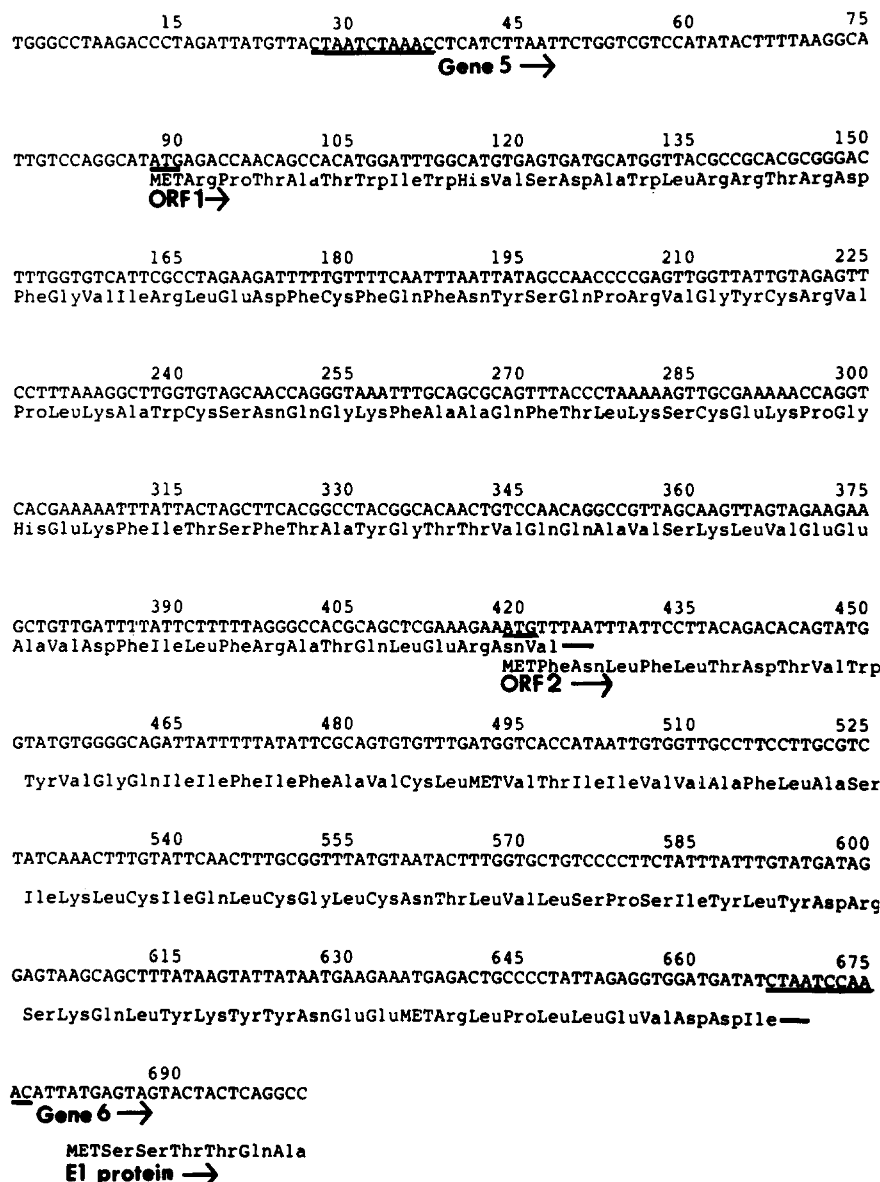

FIG. 2. Nucleotide and deduced amino acid sequence for gene 5 of MHV-A59. The DNA sequence was determined as diagrammed above and the amino acid sequence deduced using the program of Fritensky (1982). The sequence from nucleotide 1 to 701 (as numbered in Fig. 1) is shown below. The two potential open reading frames (ORF) are marked. The intergenic common sequences (Budzilowicz *et al.*, 1985), nucleotides 27–37 and 667–677, are underlined as are the putative initiator ATG sequences. The beginning of gene 6 (encoding the E1 protein) is shown as well.

cleotides 667–677) boundaries. The intergenic region at nucleotides 27–37 represents the fusion site of leader RNA to the body of mRNA 5 (Budzilowicz *et al.*, 1985; Skinner *et al.*, 1985). Within gene 5, there are ORFs which overlap by five nucleotides. The deduced amino acid sequences of these two open reading frames (deduced from the computer programs of Fritensky *et al.* (1982)) are also illustrated in Fig. 2. The first ORF predicts a polypeptide of 112 amino acids starting from the first AUG after the gene 4/5 intergenic conserved sequence and spans nucleotides 88–423. The second open reading frame (nucleotide 419–667) predicts a polypeptide of 83 amino acids. The putative initiation AUG codons for the translation of these polypeptides are both in contexts not frequently used for initiation of protein synthesis (Kozak, 1984) (see Table 1 and discussion).

To obtain more information about these putative proteins the amino acid sequences were used to determine hydropathy plots (using the analysis of Kyte and Doolittle, 1982) (Fig. 3). The larger protein (13K) contains a large proportion of basic amino acids. The smaller protein (9.6K) has a strikingly long hydrophobic region at the amino terminus followed by a basic region.

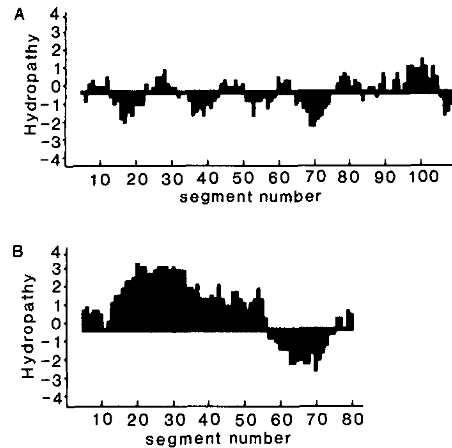

FIG. 3. Hydropathy plots for the two proteins predicted from the sequence of MHV-A59 gene 5. Hydropathy plots (Kyte and Doolittle, 1982) were derived from the amino acid sequences shown in Fig. 2. Each bar represents the average hydropathy of a segment of 9 amino acids. Each bar is placed at the midpoint of the segment. The baseline (−0.49) is the average hydropathy for most proteins that have been sequenced (9). The slashes on the abscissa are every 10 segments. (A) 13K protein, predicted from ORF 1. (B) 9.6K protein predicted from ORF 2.

Cell-free translation of MHV-A59 gene 5 RNAs

Proteins with the properties predicted from the sequence of gene 5 have been difficult to detect either in infected cells or by *in vitro* translation of MHV-A59 mRNAs purified from infected cells (Leibowitz *et al.*, 1982; Skinner *et al.*, 1985, see discussion). Thus to study the protein products of gene 5, we carried out translation in a cell-free system using large amounts of pure RNAs containing the ORFs of gene 5. This was accomplished using RNAs transcribed from pGEM vectors containing fragments of viral DNA representing gene 5, using bacteriophage RNA polymerases. Viral g344 DNA was excised from pBR322 and inserted into pGEM-1. DNA fragments containing each of the two ORFs were isolated from g344 DNA and subcloned into pGEM plasmids such that the AUG at the beginning of the ORF was the first from the 5′ end of the resulting RNA. The construction of the plasmids is described in more detail in Fig. 4 and under Materials and Methods.

RNAs were transcribed from the pGEM recombinant plasmids (as diagrammed in Fig. 4) and translated in a wheat germ cell-free translation system all as described under Materials and Methods. After translation, products were resolved on 8–16% polyacrylamide gradient gels. Figure 5 illustrates the products of cell-free translation of these RNAs as well as the protein products from translation of RNA from infected and control mock-infected 17Cl-1 cells. Infected cell RNA directed the synthesis of structural proteins N (55K) and E1 (nonglycosylated precursor of 20K) (Fig. 5), both as previously described (Leibowitz *et al.*, 1982). There was no evidence for gene 5 products in translation of this RNA. However in translation of the ORF 1 and ORF 2 RNAs there were products of the specific sizes predicted from the sequences. As shown in Fig. 5, ORF 1 directed the synthesis of a polypeptide of the size predicted from the ORF 1 sequence (9.6K) and ORF 2 RNA directed the synthesis of a polypeptide of the size predicted from the ORF 2 sequence (13K). The g344 RNA, which

TABLE 1

SEQUENCES SURROUNDING AUG INITIATION CODONS FOR MHV POLYPEPTIDES

	−3 1 4[a]
ORF 1	CAUAUGA
ORF 2	GAAAUGU
E1 protein[b]	AUUAUGA
N protein[c]	AGGAUGU
Consensus[d]	ACCAUGG (G)

[a] Nucleotides are numbered relative to the initiation codon AUG where the +1 position represents the A of the AUG.

[b] The sequence surrounding the AUG initiator codon for MHV-A59 E1 protein was taken from Armstrong *et al.* (1984).

[c] The sequence surrounding the AUG initiator codon for MHV-A59 nucleocapsid protein was taken from Spaan *et al.* (1984).

[d] The sequence most likely to be found at the site of protein synthesis initiation in eukaryotic mRNAs (Kozak, 1984).

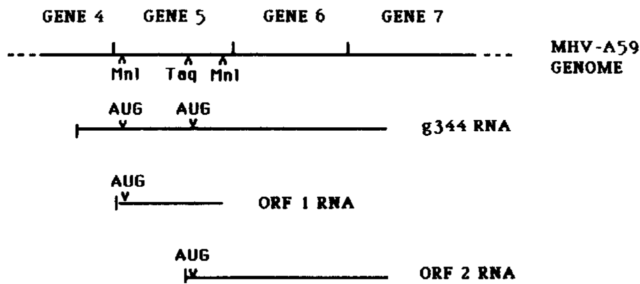

FIG. 4. Virus-specific gene 5 mRNAs transcribed from pGEM recombinant vectors. g344 DNA was excised from pBR322 by *Pst*I digestion and ligated into pGEM-1. The recombinant plasmid was digested with *Eco*RI and transcribed with SP6 polymerase; the resulting RNA is designated g344 RNA. g344 cloned DNA was digested with endonuclease *Mnl*I to excise the ORF 1 (see sites on Fig. 2) which was inserted into the *Sma*I site of pGEM-2. The plasmid was then digested with *Hin*dIII and RNA transcribed using T7 RNA polymerase; the resulting RNA is designated ORF 1 RNA. Cloned g344 was alternatively digested with *Taq*I to excise ORF 2 which was inserted into *Pst*I/*Acc*I cleaved pGem-1. This plasmid was then cleaved with *Hin*dIII and RNA transcribed with SP6 bacteriophage RNA polymerase; the resulting RNA is designated ORF 2 RNA.

contains both ORFs overlapping by five nucleotides, as in the authentic mRNA 5, directed the synthesis primarily of the 9.6K putative product of ORF 2. There was, however, a small amount of the 13K putative ORF 1 product present in the translation products of g344 RNA as well. This suggests that g344 RNA is preferentially translated from the downstream ORF.

DISCUSSION

Nucleotide sequence analysis of gene 5 of MHV-A59 illustrates the presence of two ORFs that potentially encode two nonstructural proteins, both of which are small and low in methionine content. This sequence confirms and extends the studies of Skinner *et al.*, (1985) who sequenced all of MHV strain JHM gene 5 and most of strain A59 gene 5. The upstream ORF of A59 potentially encodes a 13K Da protein with only one methionine. This protein is rich in basic amino acids and thus may be a nucleic acid binding protein and thus perhaps plays a role in viral RNA synthesis. The downstream ORF of A59 predicts a polypeptide of 9.6K with three methionines. This protein has a long stretch of hydrophobic amino acids (approximately 2/3 of the polypeptide at the amino terminus). The hydrophobicity values predicted for this protein are often associated with proteins that are embedded in membranes (Kyte and Doolittle, 1982). It may perhaps play a role in association of viral polymerase complexes with membranes. Similar proteins have been predicted from the sequence of gene 5 of the JHM strain of MHV (Skinner *et al.*, 1985). As suggested by this group, the sequence data predict differences between the proteins potentially encoded by gene 5 of JHM and A59. The predicted A59 ORF 1 product has an extra six amino-terminal amino acids while the A59 ORF 2 product lacks five carboxy-terminal amino acids relative to the predicted JHM polypeptides. It will be important to characterize these proteins during infection to determine if either is involved in determining the differences in the biological properties of the two strains.

Small nonstructural proteins have been found associated with other RNA viruses. Sequencing studies of another coronavirus, infectious bronchitis virus (IBV), predict the synthesis of two small proteins encoded by overlapping reading frames; one of these proteins is quite hydrophobic (Boursnell and Brown, 1984). It has been shown recently that other classes of RNA viruses have small hydrophobic polypeptides. For example, influenza virus has a nonstructural integral membrane M_2 protein (Zebedee *et al.*, 1985) and the paramyxovirus simian virus 5 generates a 5K Da SH protein that is extremely hydrophobic; the function and cellular location of this protein is not known (Hiebert *et al.*, 1985).

It has been extremely difficult to detect these putative

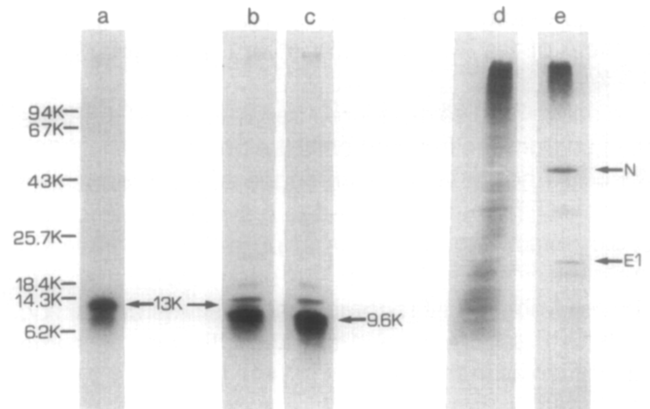

FIG. 5. Cell-free translation of mRNAs extracted from infected cells and synthesized *in vitro* from pGEM vectors. RNAs were transcribed *in vitro* from pGEM recombinant vectors as described in the legend to Fig. 4. Alternatively RNAs were extracted from infected and mock infected 17Cl-1 cells and poly(A)-containing RNA purified by oligo(dT)-cellulose chromatography. Approximately 1 μg of each of these RNAs was used to program a cell-free translation system derived from wheat germ. Translation proceeded in the presence of tritiated leucine. The products were analyzed on 8–16% polyacrylamide gradient gels. Lane a, ORF 1 RNA; lane b, g344 RNA; lane c, ORF 2 RNA; lane d, mock-infected cell RNA; lane e, infected cell RNA. Sizes for cell-free translation products were obtained by comparison of migration with marker polypeptides as designated on the left of lane a. The major products of translation of the infected cell RNA are the 55K nucleocapsid related polypeptide (designated N) and the 20K precursor of the E1 glycoprotein as seen in lane e as we have previously observed (Leibowitz *et al.*, 1982). The data shown in this figure represent one of six translation experiments all of which had essentially the same results. All the lanes shown in this figure were derived from the same gel and were spliced together to remove unnecessary lanes.

MHV proteins during infection and it is likely that there are very low concentrations of these proteins present during infection compared with other viral proteins. The only report of a possible gene 5 protein product is the finding of a 9–10K polypeptide in MHV-infected cells and in the products of translation of infected cell RNA (Skinner *et al.*, 1985). [Putative IBV nonstructural proteins predicted by sequence analysis have also been elusive (Boursnell and Brown, 1984).] The difficulty in detecting MHV gene 5 products might be explained by transcriptional and/or translational controls. MHV mRNA 5 is one of the less abundant viral mRNAs in the cell during infection (Leibowitz *et al.*, 1981). The sites surrounding the putative protein synthesis intitiation codons in gene 5, CAUAUGA for ORF 1 and GAAAUGU for ORF 2, are not those usually used for initiation of eukaryotic protein synthesis, whereas the initiator AUGs for gene 6 (E1 protein) and gene 7 (nucleocapsid protein) are in the contexts AUUAUGA and AGGAUGU (Spaan *et al.*, 1984), respectively, both of which are frequently found as functional initiation sites. The consensus sequence for initiation of protein synthesis on eukaryotic mRNAs is A(G)CCAUGG where the A (present 79% of the mRNAs) or less frequently the G (present in 18% of the mRNAs) at the −3 (relative to the A of the AUG) position is very important (Kozak, 1984).

In earlier studies of cell-free translation of MHV RNAs purified from the infected cells (Leibowitz *et al.*, 1982), we could not detect the presence of the products of these ORFs. Thus we turned to a system in which we could obtain large amounts of pure RNAs containing the gene 5 ORFs. For this we used RNA transcripts derived from pGEM recombinant plasmids containing each ORF separately and the two ORFs in the same orientation as in the genome (g344). Using this system the putative protein products were translated. The RNAs representing each of the isolated ORFs were translated into polypeptides of the expected sizes in cell-free translation systems derived either from wheat germ (Fig. 5) or rabbit reticulocyte (data not shown). The wheat germ system was much more efficient in translation of these very small polypeptides. The RNA containing both ORFs was translated primarily from the downstream ORF although there was consistently a small amount of the putative ORF 1 product translated from this RNA. The fact that ORF 2 initiator (containing a G at the −3 position) is in a better context than ORF 1 (containing a C in the −3 position) may explain why the ORF 2 product is preferentially synthesized from g344 mRNA even though the ORF 1 initiation AUG is closer to the 5′-end of the RNA.

The putative ORF 1 product is synthesized more efficiently from the ORF 1 RNA than from the g344 RNA, which contains both ORFs. This may reflect the fact that the ORF 1 initiator AUG is closer to the 5′-end on the ORF 1 RNA compared to the g344 RNA. Alternatively, sequences upstream from the ORF 1 sequence in g344 RNA (approximately 330 nucleotides) may make translation inefficient.

The generalization that eukaryotic mRNAs are monocistronic and initiate protein synthesis primarily at 5′-proximal AUGs is not always true. There are now several classes of RNA viruses known to generate mRNAs that encode two proteins in overlapping reading frames. Two examples are reovirus hemagglutinin mRNA (Ernst and Shatkin, 1985) and Sendai virus P protein mRNA (Curran *et al.*, 1986). In both these cases mRNAs contain two overlapping ORFs both of which are expressed as protein; in both cases the second ORF has an AUG in a better context for initiation of protein synthesis and is translated more efficiently. It is thought that because the first AUG is in a suboptimal context for initiation of translation, ribosomes bypass this AUG and initiate at the internal AUG. Preferential translation *in vitro* of the downstream ORF appears also to be the case for the MHV-A59 gene 5 RNA. We are investigating whether preferential translation of the ORF 2 product from mRNA 5 is reflected in the polypeptides synthesized in the MHV-A59 infected cell by raising antisera to be used to detect these proteins during infection.

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