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## Coronavirus Translational Regulation: Leader Affects mRNA Efficiency

STANLEY M. TAHARA,\*†<sup>1</sup> THERESE A. DIETLIN,\* CORNELIA C. BERGMANN,\*† GARY W. NELSON,\*  
SHIGERU KYUWA,\*<sup>2</sup> RICHARD P. ANTHONY,† AND STEPHEN A. STOHLMAN\*†

Departments of \*Microbiology and †Neurology, University of Southern California School of Medicine, Los Angeles, California 90033

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Cells infected with the murine coronavirus, mouse hepatitis virus (MHV), show decreased host protein synthesis concomitant with an increase in viral protein synthesis. We examined the *in vitro* translation property of the conserved MHV 5'-leader RNA sequence by constructing chimeric mRNAs in which the 72-nt 5'-leader of M protein mRNA (A59 strain) was positioned upstream of the human  $\alpha$ -globin coding region in a T7 expression vector. Synthetic 5'-capped transcripts of these mRNA constructs were translated in cell-free extracts prepared from uninfected and MHV-infected murine DBT cells. Nonviral mRNAs translated readily in both uninfected and infected cell-free extracts. By contrast, replacement of the human  $\alpha$ -globin 5'-untranslated region (UR) with the MHV 5'-leader increased translation ca. three- to fourfold in cell-free extracts from MHV-infected cells versus translation in extracts from uninfected cells. Chimeric globin mRNA containing the reverse complementary sequence of the viral leader RNA in the 5'-UR showed no such increase in translation, indicating sequence specificity for the effect. A 13-nt region (-UCUAAUCCAAACA-) immediately proximal to the start codon was found to be important for the increased translation of the MHV leader-containing mRNAs. These data indicate that the apparent down-regulation of host translation is not primarily due to an inhibition of host translation but also involves a significant stimulation of viral translation *in cis* by a structural feature of the MHV 5'-leader RNA sequence in conjunction with a virus-specified or virus-induced factor. © 1994 Academic Press, Inc.

### INTRODUCTION

Mouse hepatitis virus (MHV), a member of the coronavirus family (Lai, 1990), is an enveloped virus containing a single-stranded, plus (+) sense RNA genome of approximately 31 kb (Lee *et al.*, 1991). The genome is encapsidated into a helical structure composed of the phosphorylated nucleocapsid, or N protein. Virions also contain two envelope glycoproteins: an N-linked 240-kDa glycoprotein designated S and an O-linked 23- to 25-kDa glycoprotein designated M. The S protein is required for infectivity, induces cell-cell fusion, and is the target structure for neutralizing antibody (Lai, 1990). The M protein functions as a matrix protein. Some strains also express an additional envelope glycoprotein, the hemagglutininesterase or HE protein, which is not essential for replication but may play a role in viral pathogenicity (Yokomori *et al.*, 1992). Infection of susceptible cells proceeds via the synthesis of genomic-length negative-strand RNA which serves as template for transcription of the viral mRNAs (Lai, 1990). A leader RNA sequence (65-84 nt), found at the 5'-ends of genomic RNA and all subgenomic mRNAs (Lai *et al.*, 1982; Shieh *et al.*, 1987) and as a free species, is transcribed from an independent transcription unit (Lai, 1990). Although the precise mechanism of coronavirus transcription is not clear, it is

believed that at least one step involves the 3'-end of free leader binding to conserved complementary intergenic sequences on the minus (-) sense genome. The complex of free leader annealed to the intergenic sequence serves as a primer for the subsequent transcription of viral mRNAs. In addition to their common, 5'-leader-derived sequences, the viral mRNAs are structurally similar in that they form a nested set with coterminal 3'-ends (Lai, 1990). In general the 5'-most coding sequences are translated, consistent with 5'-end-dependent initiation of translation, although structurally the mRNAs are polycistronic (Siddell *et al.*, 1981a). Nonetheless, complex translation patterns have been reported for MHV. Strain A59 mRNA-5 has two functional, overlapping reading frames in the infected cell (Leibowitz *et al.*, 1988), and infectious bronchitis virus (IBV) mRNA-3 is functionally tricistronic and initiates translation of the 5'-distal open reading frame (ORF) by internal initiation (Liu and Inglis, 1992).

MHV infection results in an apparent inhibition of host-cell protein synthesis concomitant with an increase in viral protein synthesis (Siddell *et al.*, 1981b). A large relative increase in viral translation is superimposed on a background of reduced total protein synthesis in infected cells (Hilton *et al.*, 1986). This overall decline in host translation is reflected by decreased polysome size and number and an increase in inactive 80S monosomes (Hilton *et al.*, 1986). During the early stages of MHV infection there is also a decline in steady-state levels of some, but not all, host-cell mRNAs (Hilton *et al.*, 1986; Kyuwa *et al.*, submitted for publication). This would suggest that

<sup>1</sup> To whom reprint requests should be addressed.

<sup>2</sup> Present address: Department of Animal Pathology, Institute for Medical Science, University of Tokyo, Tokyo, Japan.

the decrease in global translation is partly due to host mRNA degradation. Global translational decrease commonly observed in other cytotytic virus infections is due primarily to virus-induced alterations of the translation machinery (for review, see Schneider and Shenk, 1987; Sonenberg, 1990). Dual infection experiments with MHV and vesicular stomatitis virus suggest that MHV infection does not modify host translation initiation factors (Hilton *et al.*, 1986; Baric *et al.*, 1988). The sum of the studies to date indicates, at least for MHV, that competition for active ribosomes plays a role in host cell translation shutoff early in infection, while degradation of host mRNAs assumes an increasingly important role late in infection (Hilton *et al.*, 1986).

The mechanism used by MHV to shut off host translation is intriguing since the viral mRNAs are 5'-capped and 3'-polyadenylated and thus structurally equivalent to host mRNAs. The preferential translation of viral mRNAs must involve a mechanism capable of selecting between host and viral mRNAs through some other property. The possibilities include differences in initiation factor requirements or *cis* elements which modify mRNA activity.

In this study we demonstrate that MHV mRNAs are preferentially translated in extracts from infected cells. The data indicate that the 5'-untranslated viral leader sequence functions as a *cis* augmentor of translation. This qualitative property of the viral mRNAs would account for the observed increase in viral protein synthesis during global shutoff of host translation.

## MATERIALS AND METHODS

### Virus and cells

The A59 strain of mouse hepatitis virus (MHV-A59) was used throughout (Baric *et al.*, 1988). Virus was propagated and plaque assayed in the murine astrocytoma DBT cell line as previously described (Stohman *et al.*, 1982). For monolayer cultures, cells were grown in minimum essential medium (MEM; Irvine Scientific, Irvine, CA) containing 7% heat-inactivated newborn calf serum (Irvine Scientific) and 10% tryptose phosphate broth. For suspension cultures, cells were grown in Joklik's modified MEM, containing 7% heat-inactivated newborn calf serum.

For radiolabeling experiments, DBT cells grown in 60-mm plastic petri dishes were infected at a multiplicity of infection (m.o.i.) of 10 PFU/cell. One hour prior to harvest, the medium was replaced with methionine-free MEM (Irvine Scientific). After 30 min of incubation, the medium was replaced with methionine-free medium containing 0.05 mCi/ml of [<sup>35</sup>S]methionine (Translabel; ICN, Irvine, CA). Cells were lysed in sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol], heated at 65° for 15 min and analyzed by SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels (Laemmli, 1970). Sam-

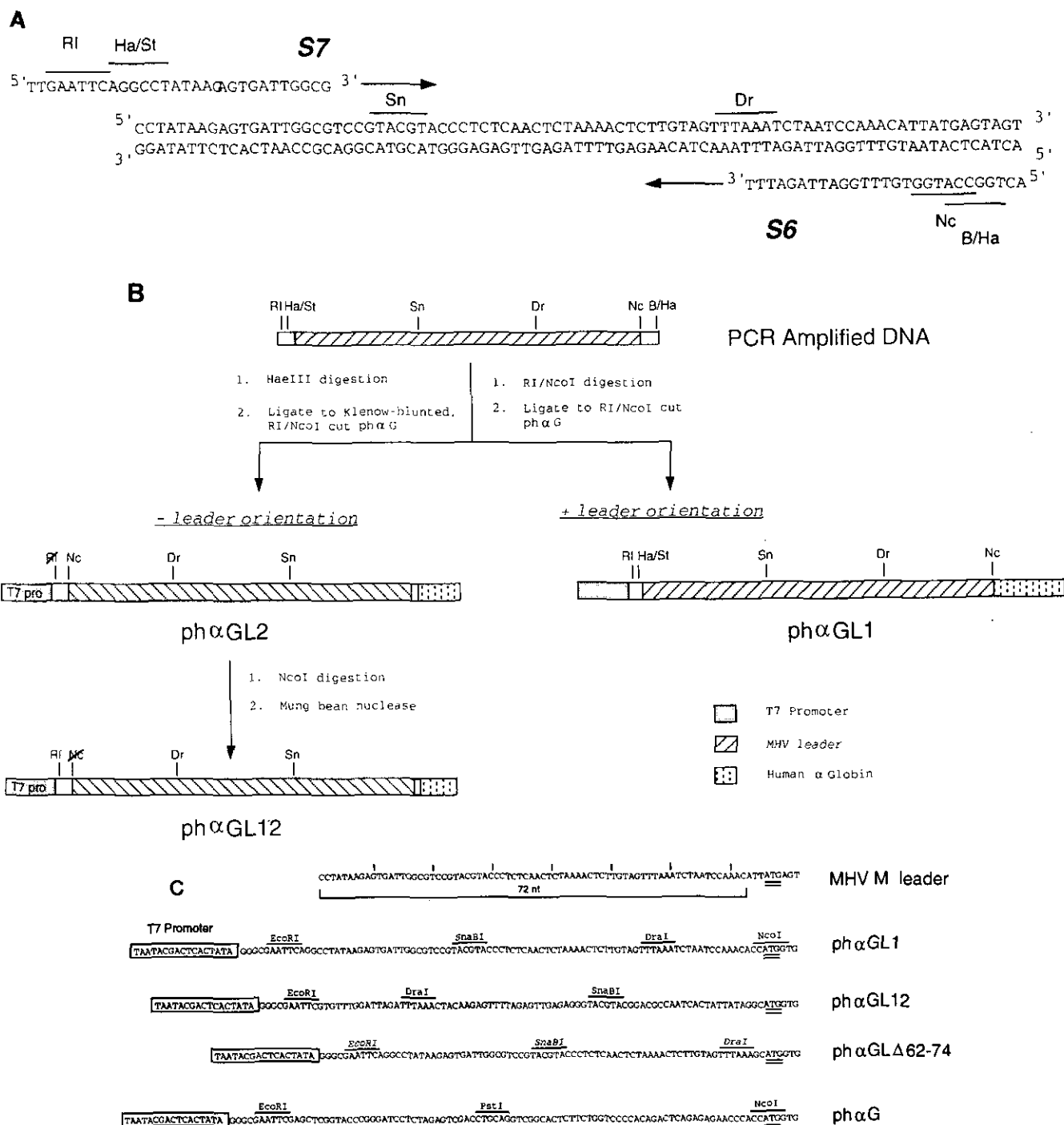
ple sizes were adjusted to equal cell equivalents for electrophoresis.

### Polymerase chain reactions (PCR)

PCR incubations were carried out in a Perkin-Elmer/Cetus DNA Thermal Cycler using standard reaction mixtures suggested by the vendor. Plasmid E1-L(+) (Stohman *et al.*, 1988) containing 90 bp of the MHV-A59 leader sequence from mRNA-6, encoding the M gene (Armstrong *et al.*, 1984), was used as a template to amplify the leader sequence. A typical reaction contained 500 ng of template and 2 μg of each primer (see below) in a 100-μl reaction volume. Samples were subjected to 30 cycles of amplification.

### Plasmids

The full-length human α-globin cDNA was subcloned into the *Pst*I site of pGEM3Zf(-) by insertion of the 0.6-kb *Pst*I/*Pst*I fragment from pSPhαFL (obtained from Dr. S. Liebhaber, University of Pennsylvania). The resulting plasmid was designated phαG and contains the entire mRNA sequence, including 18 adenylate residues of the 3'-poly(A) tail, 5'-UR of 37 nt, and the complete 3'-UR, (see Fig. 1C for 5'-UR sequence). The 5'-untranslated region of α-globin mRNA was replaced by various MHV sequences. The MHV leader was obtained by PCR amplifying (Scharf *et al.*, 1986) the 5'-UR of the A59 M mRNA using the primers: S6 (5'-TTG AAT TCA GGC CTA TAA GAG TGA TTG GCG-3') and S7 (5'-ACT GGC CAT GGT GTT TGG ATT AGA TTT-3'). Both were designed to include restriction sites to facilitate cloning (see Fig. 1A). The amplified 106-bp fragment was cleaved with *Eco*RI and *Nco*I, gel purified, and ligated into *Eco*RI/*Nco*I-cleaved phαG. The resulting plasmid construction (phαGL1) contained the MHV leader DNA in the positive (+) sense with respect to the T7 promoter and coding region (Figs. 1B and 1C). Construction of phαGL2 was carried out analogously (Fig. 1B), except that the PCR fragment was cleaved with *Hae*III to generate blunt ends. This fragment was ligated into phαG whose *Eco*RI/*Nco*I cut ends had been blunt-ended with Klenow polymerase. The blunt ending introduced a second initiation codon (within the *Nco*I site) upstream of the leader sequence in phαGL2 (Fig. 1B). The upstream start codon was deleted by cleavage with *Nco*I and treatment with mung bean nuclease (8 U/μg DNA; Boehringer, Indianapolis, IN) for 10 min at 30° (Hasan *et al.*, 1986). Religation yielded the desired construct designated phαGL12 with the leader in antisense preceding the human α-globin gene. During construction of phαGL12, a construct was also isolated with (+) sense leader but with a deletion of nucleotides 62-74 (base positions in native M mRNA). This construct was designated phαGLΔ62-74. All constructions were sequenced from ds plasmid DNA using the method of Zagursky *et al.* (1985).



**Fig. 1.** Construction of MHV leader-human  $\alpha$ -globin chimeric genes. (A) PCR site-directed mutagenesis of MHV leader. The sequence of DNA amplified from mRNA-6 cDNA (M gene) clone by PCR is depicted. The relative positions of the amplification primers (S6 and S7) are shown, including their sequences. Note that the resulting amplified DNA contained an *NcoI* site at the native ATG start codon. Primer S6 converted the viral ATG into an *NcoI* site and introduced *BalI* and *HaeIII* restriction sites. Primer S7 introduced *EcoRI*, *HaeIII*, and *StuI* sites in the amplified DNA. RI, *EcoRI*; Ha, *HaeIII*; St, *StuI*; Sn, *SnaBI*; Dr, *DraI*; Nc, *NcoI*; B, *BalI*. (B) Construction strategy for MHV leader-human  $\alpha$ -globin chimeric genes. Two approaches were used to construct MHV leader-human  $\alpha$ -globin chimeric genes. For construction of the (+) sense leader with a human  $\alpha$ -globin coding region (ph $\alpha$ GL1), the PCR amplified DNA was treated with *EcoRI* and *NcoI* and inserted into the correspondingly treated ph $\alpha$ G plasmid. For the (-) leader constructions (ph $\alpha$ GL2 and ph $\alpha$ GL12), *EcoRI/NcoI*-digested ph $\alpha$ G was Klenow-treated and ligated to *HaeIII*-digested, PCR-amplified DNAs. The intermediate product (ph $\alpha$ GL2) was treated with *NcoI* and mung bean nuclease to remove the introduced *NcoI* site (C<sup>-</sup>CATGG). During construction of ph $\alpha$ GL2, an isolate containing the MHV leader in the (+) orientation with a deletion of nucleotides 62-74 was isolated (ph $\alpha$ GL $\Delta$ 62-74). Plasmids ph $\alpha$ G, ph $\alpha$ GL1, ph $\alpha$ GL12, and ph $\alpha$ GL $\Delta$ 62-74 were used for translation studies. (C) MHV leader-human  $\alpha$ -globin mRNA constructions. The MHV leader-human  $\alpha$ -globin constructions used in this study are depicted. Translation initiation sites are denoted by a double underscore. For comparison, the authentic MHV M gene leader sequence is shown at the top, including the relative position of the authentic translation initiation site in this mRNA. The 5'-end sequence of the parental h $\alpha$ G plasmid is shown at the bottom.  $\alpha$ -Globin coding sequences following the start codon are identical among all constructions. All constructions were verified by dideoxynucleotide sequencing of ds plasmid DNAs (Zagursky *et al.*, 1985). Restriction site positions are denoted by bars with the corresponding identity of the recognition sequence. For transcription, the cap site corresponds to the G residue immediately 3' of the T7 promoter (boxed).

### Preparation of poly(A) mRNA

Total cellular RNA was isolated from MHV-infected cells as previously described (Lai *et al.*, 1982) except that cells were not treated with actinomycin D. The washed and dried RNA pellet was dissolved in sterile glass-distilled water. Globin mRNA was isolated, as previously described, from rabbit reticulocytes (Grifo *et al.*, 1982) harvested from phenylhydrazine-treated New Zealand White rabbits (Tahara *et al.*, 1981).

### *In vitro* transcription

Plasmids were linearized with *Sph*I and purified prior to *in vitro* transcription as previously described (Darzynkiewicz *et al.*, 1988; Tahara *et al.*, 1991). 5'-Capped RNA transcripts were synthesized in a 50- $\mu$ l reaction volume containing: 40 mM Tris-HCl (pH 7.9), 8 mM MgCl<sub>2</sub>, 2 mM spermidine, 5 mM DTT, 0.5 mM NTPs (except GTP), 0.1 mM GTP, 1.0 mM m<sup>7</sup>GpppG (New England Biolabs), 1000 U/ml RNasin (Promega Biotec), 100  $\mu$ g/ml linearized plasmid, 7.5  $\mu$ Ci [<sup>3</sup>H]ATP, and 400 U/ml T7 RNA polymerase (Promega Biotec). Mixtures were incubated for 30 min at 37°, followed by a second aliquot of enzyme and additional incubation for 30 min. Template DNA was digested after transcription with 5  $\mu$ g/ml DNase I at 37° for 10 min. This mixture was extracted with phenol:CHCl<sub>3</sub>:isoamyl alcohol (25:24:1, v/v/v) and CHCl<sub>3</sub>:isoamyl alcohol (24:1, v/v). Transcripts were separated from unincorporated nucleotides and oligo DNA by precipitation from 2.5 M NH<sub>4</sub>OAc with 2.5 vol of ethanol at -20°. RNA was collected by centrifugation for 25 min (13,200 g) at 4°, washed in 70% ethanol, dried, resuspended in sterile water, and reprecipitated from 2.5 M NH<sub>4</sub>OAc as above. Twice-precipitated RNA was resuspended in 25  $\mu$ l of sterile water. An aliquot was added to Safety Solve (Research Products International, Mount Prospect, IL) and quantitated by liquid scintillation spectrometry to determine product yield and concentration. Transcript solutions were stored at the vapor temperature of liquid nitrogen. Transcripts are denoted in the text by the plasmid name without the plasmid prefix.

### *In vitro* translation

For the preparation of translation extracts from infected cells, DBT cells were grown in 150-mm plastic petri dishes (Falcon Plastics, Oxnard, CA) to confluency and infected at an m.o.i. of 1 PFU/cell. Cells were harvested at 6 hr postinfection (p.i.). At the time of harvest, cells exhibited approximately 40–60% cytopathic effect (see below). Uninfected DBT cells were grown in suspension culture and cell-free translation extracts were prepared as previously described (Rose *et al.*, 1978; Tahara *et al.*, 1981, 1991). For preparation of cell-free extracts from monolayers the same protocol was used with the following modifications. Medium was aspirated and replaced with ice-cold isotonic buffer [35 mM Hepes (pH

7.5), 146 mM NaCl, 11 mM glucose; 5 ml per 150-mm dish]. Cells were scraped directly into this buffer and collected by centrifugation. The cell pellets were resuspended and washed three times in isotonic buffer as described before and lysed in 1 vol of hypotonic buffer [10 mM HEPES (pH 7.5), 15 mM KCl, 1.5 mM Mg(OAc)<sub>2</sub>]. Lysates were preincubated to dissociate polysomes into free ribosomal subunits as described (Tahara *et al.*, 1991). Prior to use, extracts were treated with 125 U/ml micrococcal nuclease at 21° for 0–12 min. Optimum incubation times were determined for each extract preparation. *In vitro* translation incubations were performed for 20 or 60 min in a 15- $\mu$ l vol containing 9  $\mu$ l of nuclease-treated cell extract and 15  $\mu$ Ci [<sup>35</sup>S]methionine (Amersham, Arlington Heights, IL) as previously described (Tahara *et al.*, 1991). Synthetic, capped mRNAs were added to the incubation mixture at a concentration of 16.7  $\mu$ g/ml. Extracts maintained linear translation activity with exogenous mRNA concentrations up to 100  $\mu$ g/ml mRNA (data not shown). Incubation mixtures were precipitated by addition of 1 ml of cold acetone to terminate the reaction. Precipitates were collected by centrifugation, resuspended in Laemmli sample buffer, and analyzed on 15% polyacrylamide gels under denaturing conditions (Laemmli, 1970). Dried gels were exposed to XAR-5 X-ray film (Kodak) and autoradiographed for 1–14 days. Polypeptide bands were quantitated using a Hoefer scanning densitometer and integrated gravimetrically.

### RNA isolation and Northern blot analysis

Translation mixtures were terminated with 200  $\mu$ l of cold Solution 1 [10 mM Tris-HCl, (pH 7.5) 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.65% NP-40]. After 5 min on ice, particulates were removed by centrifugation at 4° and the supernatant was combined with 1 vol of Solution 2 (7 M urea, 1% SDS, 350 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA). The mixture was digested with 5  $\mu$ l of 10 mg/ml Proteinase K for 15 min at 37° followed by extraction with phenol:CHCl<sub>3</sub>:iAmOH (25:24:1) and CHCl<sub>3</sub>:iAmOH (24:1). RNA was precipitated from 0.3 M NaOAc (pH 6), with 2.5 vol ethanol at -20°, collected by centrifugation at 4°, and washed with 75% ethanol before drying. All solutions used for RNA isolation prior to RNA transfer were treated with diethylpyrocarbonate. RNA samples were prepared and resolved on a 1% formaldehyde-formamide agarose gel as described (Fourney *et al.*, 1988) except that they were first dissolved in 3  $\mu$ l of 25 mM EDTA, 0.1% SDS, and brought to 18  $\mu$ l with 15  $\mu$ l Electrophoresis Sample Buffer. RNA was transferred to Biotrans nylon membrane (ICN) by rapid downward alkaline transfer (Chomczynski, 1992). Messenger RNAs were detected via a nonisotopic hybridization detection method (NEN Renaissance), using fluoresceinated, human  $\alpha$ -globin coding region probe. Hybridization reactions and detection were performed according to the vendor's recommendations. Quantitation was done by densitometry and gravimetric integration of hybridization intensities.

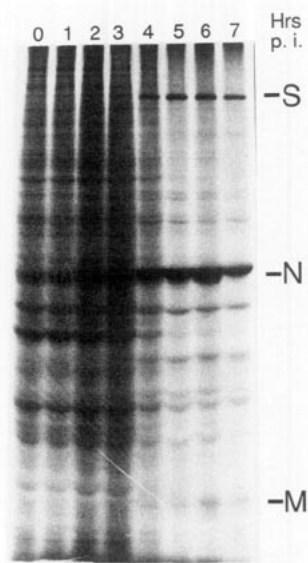


FIG. 2. Appearance of viral proteins during time course of virus infection. DBT cells were infected with MHV strain MHV-A59 at an m.o.i. of 10. At the times shown, cells were pulse-labeled with [<sup>35</sup>S]methionine and total cellular proteins were isolated and separated by SDS-polyacrylamide gel electrophoresis followed by autoradiography; the autoradiogram is shown. Equal masses (cell equivalents) were applied to each lane. The positions of viral structural polypeptides (S, N, and M) are indicated.

## RESULTS

### Time course of viral protein synthesis

Cells infected with cytotytic viruses characteristically exhibit a time-dependent decline in host-specific protein synthesis. Viral polypeptides accumulate due to preferential synthesis (Schneider and Shenk, 1987; Siddell *et al.*, 1981a,b; Sonenberg, 1990). Although the net result is similar, viruses employ different mechanisms to inhibit or suppress host translation (Schneider and Shenk, 1987). Increased MHV protein synthesis is believed to result from selective depletion of cytoplasmic mRNAs and diversion of translation components to viral transcripts. A general decline in host mRNAs was first inferred from the observation that *actin* mRNA levels fell to less than 10% of that detected in mock-infected cells (Hilton *et al.*, 1986). More recently, we observed that host mRNAs were not equally sensitive to degradation during infection (Kyuwa *et al.*, submitted for publication); therefore, global degradation of host mRNAs cannot account completely for the apparent shutoff of host mRNA translation in MHV-infected cells. We observed that MHV infection in DBT cells was accompanied by a decline in the total translation activity. An analysis of the newly synthesized viral proteins in equal cell equivalents (Fig. 2) showed that viral proteins could be detected by 3–4 hr p.i. and were predominant by 6 hr p.i., at which time total translation activity of the infected cell had declined approximately 80%. These kinetics in DBT cells are comparable to earlier reports using L-929-infected cells (Mizzen *et al.*, 1987) and are consistent with observations in other MHV-in-

ected cells (Kyuwa *et al.*, submitted for publication; Siddell *et al.*, 1981a). It is noteworthy that the maximum accumulation of viral mRNAs under these conditions does not take place until 6–7 hr p.i. in MHV-infected DBT cells (Lai *et al.*, 1981), well after the first appearance of viral polypeptides.

### Translation in cell-free extracts from infected and uninfected cells

Cell-free translation extracts from MHV-infected cells were compared to extracts from uninfected cells for translation of exogenous mRNAs (Fig. 3). Rabbit reticulocyte mRNA (predominantly  $\alpha$ - and  $\beta$ -globin) translated

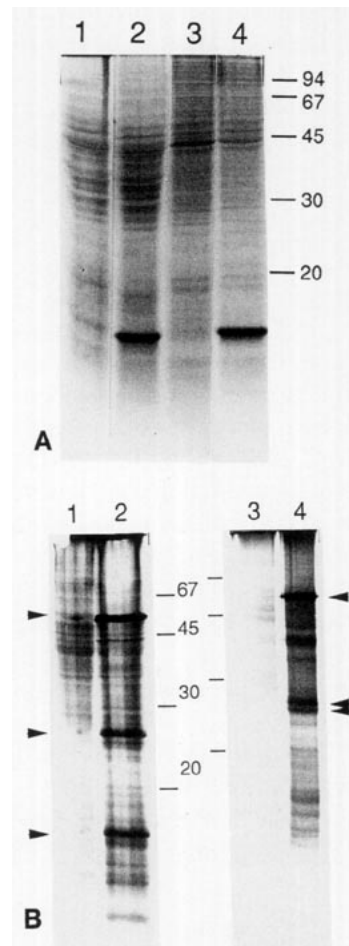


FIG. 3. Translation of MHV-A59 and globin mRNAs in uninfected and infected cell-free extracts. (A) Rabbit globin mRNA was isolated as described and assayed for translation activity in uninfected (lanes 1 and 2) or infected (lanes 3 and 4) cell-free extracts. Translation products were analyzed by SDS gel electrophoresis and autoradiography. The autoradiogram is shown. Lanes 1 and 3, no mRNA; lanes 2 and 4, globin mRNA (100 ng). (B) Oligo(dT)-selected RNA isolated from MHV-A59-infected DBT cells was assayed for translation activity in uninfected (lanes 1 and 2) or infected (lanes 3 and 4) cell-free extracts. Lanes 1 and 3, no mRNA; lanes 2 and 4, poly(A) mRNA added (2.5  $\mu$ g). Numbers to the right of the panel indicate positions of molecular size standards (in kDa): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa). Inset arrows indicate positions of major viral proteins.

efficiently in both uninfected (Fig. 3A, lane 2) and infected (Fig. 3A, lane 4) cell-free extracts, giving the expected single band of mixed globins. Synthetic, capped chloramphenicol acetyltransferase mRNA also translated efficiently in both systems (data not shown). These results indicated that MHV-infected cells do not lose the ability to translate either cellular or synthetic capped mRNAs. This result contrasts with the shutoff mechanism observed in extracts of poliovirus infected cells, where eukaryotic initiation factor-4F (eIF-4F) is inactivated, resulting in an inability to translate capped mRNAs (Sonenberg, 1990).

As shown in Fig. 3B (lane 4), the major polypeptide resulting from translation of oligo(dT)-selected mRNA in infected cell-free extract was the viral N protein (55 kDa), similar to the translation patterns reported earlier (Siddell *et al.*, 1981b). An M protein doublet was also observed; identity of the doublet was confirmed by immunoprecipitation with anti-M antibody (not shown). By comparison, addition of intracellular viral mRNAs to cell-free extracts from uninfected cells yielded the viral N protein, M protein, and a 16-kDa polypeptide (see Fig. 3B, lane 2). The identity of the 16-kDa translation product is unknown. Recent data suggest that an intergenic consensus sequence within gene 7 may result in a novel mRNA predicted to yield a 16-kDa polypeptide (Baric, personal communication); however, no corresponding leader containing mRNA was detected by Northern blot analysis (data not shown). Alternatively the 16-kDa protein may have arisen from an internal initiation event on mRNA-7, known to occur in DBT cell extracts (Tahara *et al.*, 1991). The qualitative difference in the translation behavior of viral mRNAs in infected and uninfected cell-free extracts led us to further examine the properties of MHV mRNAs.

#### Effect of leader on viral mRNA translation

From the general pattern of the MHV shutoff of host translation, combined with the results of the previous experiment, we hypothesized that a property of the viral mRNAs was responsible for their preferred translation

TABLE 1

RELATIVE TRANSLATION ACTIVITY OF MHV/HUMAN  $\alpha$ -GLOBIN mRNAs

mRNA	Uninfected cell extract	Infected cell extract	$\frac{\text{Infected}}{\text{Uninfected}}$
h $\alpha$ G	1	1	1
h $\alpha$ GL1	3.8	10.3	2.7
h $\alpha$ GL12	2.9	2.5	0.9
h $\alpha$ GL $\Delta$ 62-74	1.9	2	1

*Note.* MHV leader-human  $\alpha$ -globin mRNAs were translated in infected and uninfected cell-free extracts. Data from Fig. 4 were normalized to translation activity of  $\alpha$ -globin mRNA (h $\alpha$ G) in uninfected and infected cell extracts, respectively. Ratios of the normalized data are shown in the last column.

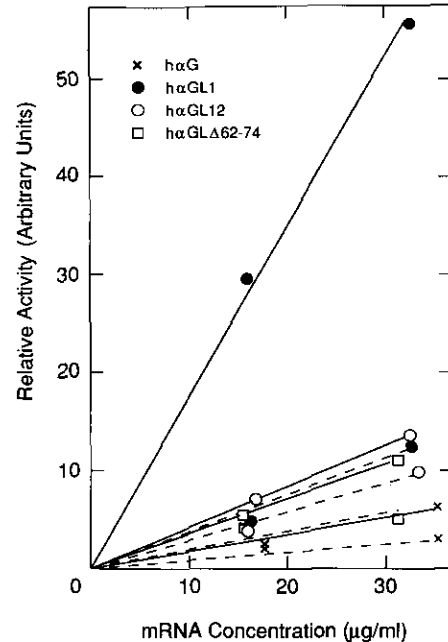
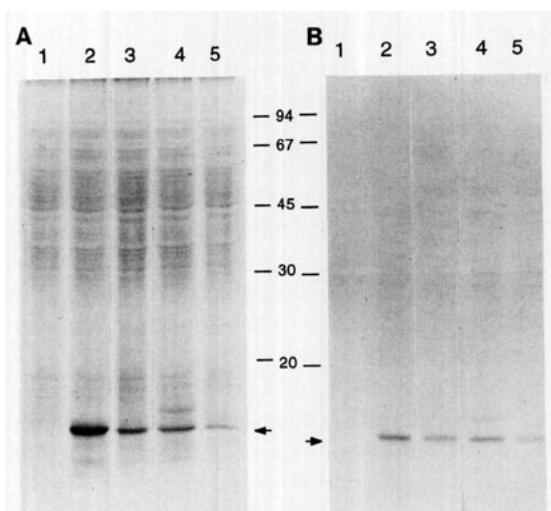


Fig. 4. Relative activity of MHV leader-human  $\alpha$ -globin mRNAs. Synthetic mRNAs were prepared from the MHV leader-human  $\alpha$ -globin constructions described in Fig. 1C. Messenger RNAs are denoted by the plasmid name less the plasmid prefix. Chimeric mRNAs were assayed in infected and uninfected cell-free extracts for relative translation activity at the indicated concentrations and under standard conditions. Products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The autoradiogram was quantitated by densitometry and the data are expressed as arbitrary density units. h $\alpha$ G (x), h $\alpha$ GL1 (●), h $\alpha$ GL12 (○), h $\alpha$ GL $\Delta$ 62-74 (□); (---) translation in uninfected extract; (—) translation in infected extract.

in infected cells. The 5'-leader sequence is an element common to all MHV mRNAs (Lai, 1990; Lee *et al.*, 1991) and thus a candidate for a specific, global *cis* mechanism affecting translation of viral mRNAs. In order to further explore this possibility the translational properties of the MHV leader were examined in isolation from other viral coding sequences by making heterologous gene constructions containing the viral leader and human  $\alpha$ -globin coding region (Fig. 1C). This reporter was chosen for ease of construction of 5'-untranslated region variants as well as the small size of the resulting protein product after *in vitro* assay. The  $\alpha$ -globin coding sequence is not subject to any translational regulation *in vivo* (Phillips *et al.*, 1977) and thus serves as a "neutral" reporter sequence in translation.

The leader  $\alpha$ -globin mRNAs and the human  $\alpha$ -globin control mRNA were titrated into infected and uninfected DBT extracts. As shown in Fig. 4, all the mRNAs were more active in the infected cell extract than in the uninfected cell extract; however, when these data were normalized to the activity of h $\alpha$ G, the activity of the h $\alpha$ GL1 mRNA, containing the MHV leader sequence, differed significantly from the other mRNA constructs in the cell-free extract from infected cells. As summarized in Table 1, h $\alpha$ GL1 was stimulated approximately threefold when translated in infected cell-free extract. The other mRNAs



**Fig. 5.** Translation of MHV leader-human  $\alpha$ -globin chimeric mRNAs. Translation conditions employed were as described; 250 ng of each synthetic mRNA was used in the translation mixture (16.7  $\mu$ g/ml final concentration) and incubation was at 37° for 60 min. Translation products were analyzed as described above. The autoradiogram is shown. Numbers in the center of the figure indicate positions of molecular size standards as described in the legend to Fig. 3. Numbers in parentheses after each mRNA represent relative expression of  $\alpha$ -globin with respect to h $\alpha$ G mRNA control (lane 5) which was arbitrarily set to 1.0 in each panel. (A) Translation in infected DBT cell-free extract. Lane 1, no mRNA; lane 2, h $\alpha$ GL1 (12.3); lane 3, h $\alpha$ GL $\Delta$ 62-74 (3.8); lane 4, h $\alpha$ GL12 (3.6); lane 5, h $\alpha$ G mRNA (1.0). (B) Translation in uninfected DBT cell-free extract. Lane 1, no mRNA; lane 2, h $\alpha$ GL1 (3.3); lane 3, h $\alpha$ GL $\Delta$ 62-74 (1.4); lane 4, h $\alpha$ GL12 (2.9); lane 5, h $\alpha$ G mRNA (1.0). The arrows indicate the migration positions of human  $\alpha$ -globin in each polyacrylamide gel.

showed no such increase in translation or slightly declined in relative activity in this experiment. From these results several conclusions can be drawn: (1) The relative activities of all synthetic mRNAs were maintained up to a concentration of 35  $\mu$ g/ml. Thus, the observed activities are intrinsic to the mRNAs. (2) Replacement of the native  $\alpha$ -globin 5'-UR with various forms of the viral leader resulted in a general increase in both uninfected and infected cell extracts. However, the h $\alpha$ GL1 mRNA was specifically stimulated when translated in the infected cell extract. (3) The activities of h $\alpha$ GL12 and h $\alpha$ GL $\Delta$ 62-74 versus h $\alpha$ GL1 implied that the (+) sense leader primary sequence is required for the increase in translation and furthermore that nucleotides 62–74, inclusive, are important in this process.

The products of the MHV leader  $\alpha$ -globin gene constructions were analyzed after translation in infected and uninfected DBT cell extracts. Figure 5 demonstrates that the accumulation of  $\alpha$ -globin correlated with the kinetic results (Fig. 4). The mRNA h $\alpha$ GL1, which contained the MHV leader in the (+) sense was approximately 4-fold more active when translated in the infected cell extract (Fig. 5A, lane 2 vs. Fig. 5B, lane 2). By comparison, the (–) sense MHV leader mRNA h $\alpha$ GL12 showed a small relative stimulation (1.2-fold) when translated in infected cell extract (Fig. 5A, lane 3 vs. Fig. 5B, lane 3). Similar to

h $\alpha$ GL12, the deletion construction h $\alpha$ GL $\Delta$ 62-74 showed a small (2.7-fold) increase in activity when translated in infected cell extract (Fig. 5A, lane 4 vs. Fig. 5B, lane 4). These results clearly show that the 72-nt MHV leader sequence confers an orientation-dependent, increased translatability of mRNA in *cis* when translated in cell-free extracts from infected but not uninfected cells. Furthermore, the activity of h $\alpha$ GL $\Delta$ 62-74 mRNA suggests that sequences important for the "augmentation" effect are contained within this region. These data are included in Table 2, which is a compilation of the results from three independent experiments. These data underscore the specific stimulatory activity in infected cell extracts of the intact MHV leader present in the sense orientation including nucleotides 62–74.

The (–) sense leader mRNA (h $\alpha$ GL12) reproducibly directed synthesis of a second polypeptide with an apparent size of 16 kDa (note: this polypeptide is not the same as the previous 16-kDa polypeptide synthesized in the presence of total viral mRNA, Fig. 3A). An ORF analysis of h $\alpha$ GL12 mRNA showed that the only functional ORF was that for  $\alpha$ -globin. An N-terminal extension of the  $\alpha$ -globin ORF is present and can potentially encode a polypeptide of 161 amino acids, comparable in size to the observed 16-kDa product. The 16-kDa polypeptide was immunoprecipitated by polyclonal anti-hemoglobin antibody, which indicates that it contains globin epitopes (data not shown). However, this ORF does not begin with an AUG codon but instead with ACU (Thr). Although non-AUG initiation of translation has been described in eukaryotes, only GUG, CUG, and ACG (Hann *et al.*, 1988; Mehdi *et al.*, 1990) or AUU (Xiao *et al.*, 1991) have been reported as potential start codons. None of these codons are present in the correct location to account for the large ORF in h $\alpha$ GL12; therefore, we are unable to explain initiation of the 16-kDa polypeptide from this mRNA.

In order to eliminate the possibility that differences in mRNA stability were responsible for the observed differ-

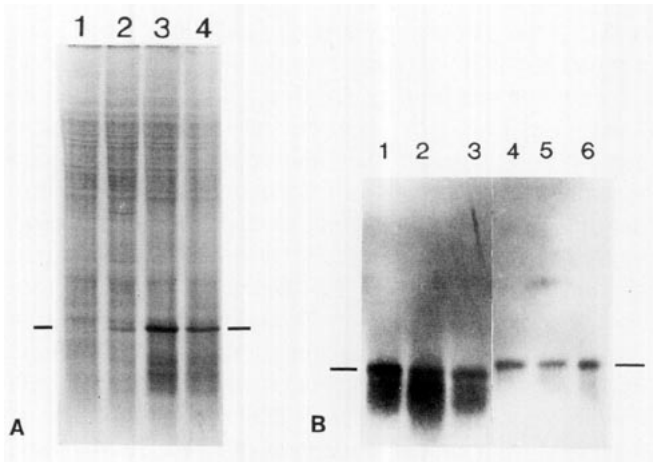
**TABLE 2**

ACTIVITIES OF MHV/HUMAN  $\alpha$ -GLOBIN mRNAs IN CELL-FREE EXTRACTS PREPARED FROM UNINFECTED AND INFECTED DBT CELLS

mRNA	Uninfected cell extract	Infected cell extract	$\frac{\text{Infected}}{\text{Uninfected}}$
h $\alpha$ G	1	1	1
h $\alpha$ GL1	2.70 $\pm$ 0.60	10.31 $\pm$ 1.70	3.8
h $\alpha$ GL12	2.38 $\pm$ 0.65	2.94 $\pm$ 0.68	1.2
h $\alpha$ GL $\Delta$ 62-74	1.63 $\pm$ 0.35	3.39 $\pm$ 0.37	2.1

*Note.* The MHV leader-human  $\alpha$ -globin mRNAs were translated in uninfected and infected cell-free extracts as described. Translation products were resolved by SDS-polyacrylamide gel electrophoresis and quantitated by autoradiography and densitometry. The data were normalized to translation activity of  $\alpha$ -globin mRNA (h $\alpha$ G) in uninfected and infected cell extracts, respectively. Results from three independent experiments are presented as the mean  $\pm$  SD. Ratios of the normalized data are shown in the last column.





**FIG. 6.** Integrity of MHV-globin mRNAs after translation assay. Synthetic MHV- $\alpha$ -globin mRNAs were translated in parallel in MHV-infected cell-free extract for 20 min at 37° as described above. One set was assayed for translation activity of the added mRNAs and the other set was analyzed by Northern blotting to assess mRNA integrity. (A) Translation activity of MHV- $\alpha$ -globin mRNAs.  $^{35}\text{S}$ -labeled  $\alpha$ -globin was detected as described above. Lane 1, no mRNA; lane 2, h $\alpha$ G; lane 3, h $\alpha$ GL1; lane 4, h $\alpha$ GL12. The lines at each side of the autoradiogram denote the position of  $\alpha$ -globin. Relative expression levels after quantitation of  $\alpha$ -globin synthesized from each mRNA: h $\alpha$ G — 1.0; h $\alpha$ GL1 — 6.0; h $\alpha$ GL12 — 3.0. (B) Northern blot analysis of recovered mRNAs. Lanes 1–3, after translation assay. Lanes 4–6, MHV- $\alpha$ -globin mRNAs, no incubation; 50 ng/lane. Lanes 1 and 4,  $\alpha$ -globin; lanes 2 and 5, h $\alpha$ GL1; lanes 3 and 6, h $\alpha$ GL12.

ences in translation, translation mixtures were analyzed by Northern blot hybridization after assay to assess the extent of mRNA degradation. Duplicate translation reactions for each mRNA were assayed for translation activity (Fig. 6A) and postassay mRNA content (Fig. 6B). The level of translation observed from h $\alpha$ GL12 was threefold higher than the control h $\alpha$ G, but in this instance, only half the activity of h $\alpha$ GL1. All synthetic mRNAs showed degradation after incubation for 20 min (Fig. 6B, lanes 1–3). Preferential degradation of h $\alpha$ GL12 did not occur; instead, h $\alpha$ GL1 showed slightly more degradation than the former. Thus, the observed differences in translation activities are not the result of differences in mRNA stability but are instead intrinsic to the 5'-UR.

## DISCUSSION

The data presented indicate that the MHV 5'-leader increases translation of a downstream coding region ( $\alpha$ -globin) only in MHV-infected cell-free extracts. This effect required the intact primary sequence since a loss in stimulatory activity was observed for chimeric  $\alpha$ -globin mRNAs with the 5'-UR replaced with the reverse complementary sequence of the MHV leader (h $\alpha$ GL12) or with a partially deleted MHV leader (h $\alpha$ GL $\Delta$ 62-74). The latter result is intriguing since nucleotides 62–74 of the M mRNA leader include the tandemly repeated pentamer motif: -UCUAAUCCAA-. The pentamer motif is tandemly repeated in the intergenic regions 5' of each gene in the viral genome and is important in the viral leader primed

transcription mechanism (Shieh *et al.*, 1987; Pachuk *et al.*, 1989; Lai, 1990). The data in this report indicate that this sequence also has a function in translation as discussed below.

The stimulatory effect of the MHV leader in translation is consistent with a *cis* recessive effect of the leader and not a *cis* dominant effect. If activation of MHV mRNA was due to a *cis* dominant property, then RNA secondary structure would be expected to play an important role. For example, a stable RNA secondary structure, normally inhibitory to initiation, would be responsible for the observed activity of the viral 5'-UR in uninfected cell extracts. This structure, upon destabilization in infected cell extract, would no longer impede initiation, thus allowing translation to proceed at a maximum rate; however, this possibility appears to be unlikely. Secondary structures with stability of  $\Delta G^\circ \leq -50$  kcal/mol act as barriers to scanning ribosomes (Pelletier and Sonenberg, 1985). Structural analysis of the MHV leader indicates an absence of stable secondary structure under physiological conditions (Shieh *et al.*, 1987; Pachuk *et al.*, 1989). A similar analysis of full-length chimeric mRNA sequences showed no stable structures which could impede 43S ribosome attachment or movement. Intrinsic structural differences cannot be the reason for the observed translation behavior of the chimeric mRNAs.

Thus, we conclude that augmentation of h $\alpha$ GL1 translation in infected cell extract operates via a *cis* recessive mechanism of which there are two possibilities. Uninfected cells could have an mRNA specific repressor which suppresses activity of MHV leader containing mRNAs. Upon infection, this repressor would either be displaced or converted to an inactive form, thus permitting unimpeded translation of viral mRNAs. When we addressed this by reconstituting MHV-infected cell extract with partially fractionated ribosomal salt wash from rabbit reticulocytes we saw no inhibition of translation of MHV leader-containing mRNAs. Instead, salt wash fractions nonspecifically stimulated translation of MHV-globin mRNAs; these results are not consistent with a repressor model. We favor the second alternative of a *cis* recessive mechanism where a viral gene product is responsible for activating MHV leader-containing mRNAs in *trans*. This preference is based on concurrent studies regarding the function of the viral N protein and its high affinity for sequences within the viral 5'-leader (see below). This mechanism contrasts with a previously identified *trans*-acting protein which binds to the iron-responsive elements (IRE) in ferritin mRNA; in this example, binding of the cognate IRE-binding protein inhibits translation (Kuhn and Hentze, 1992).

Interactions between a *cis* element and a *trans*-acting factor have been demonstrated for the translation of other viral mRNAs. For example, cellular proteins bind to sequences within the 5'-UR of poliovirus and EMCV mRNAs. For the former, a 52-kDa protein, identified as the La antigen (Meerovitch *et al.*, 1993) binds to an oligo-

pyrimidine tract in the 5'-UR (Pestova *et al.*, 1991); others observe that no fewer than three cellular proteins bind specifically to different regions of the 5'-UR of poliovirus (Gebhard and Ehrenfeld, 1992). The La protein is present at different levels in reticulocytes and HeLa cells, suggesting a possible reason for the observed difference in translation activity of poliovirus mRNA in the two systems (Meerovitch *et al.*, 1993). Recently, Furuya and Lai (1993) showed that specific proteins in uninfected cell extracts bind to the MHV leader; these proteins were proposed by them to have functions in viral replication or translation events. Thus, any proposed MHV mechanism should account for these cellular proteins.

Our results support a model requiring an activity supplied in *trans*. As discussed above this activity is present only in infected cells and requires an intact MHV leader sequence. Our current hypothesis is that the *trans*-acting factor in MHV-infected cells is the N protein. In addition to its structural function in formation of the nucleocapsid, the N protein binds specifically ( $K_d \sim 10^{-8} M$ ) to both free leader RNA found in the cytoplasm of infected cells and leader RNA covalently linked to viral mRNAs (Baric *et al.*, 1988). Previous studies demonstrated that the tandemly repeated pentamer region of the leader is important for the binding of N protein (Stohlman *et al.*, 1988). More recent studies indicate that the pentamer repeat unit alone is sufficient to serve as a ligand for the RNA binding site of N protein (unpublished data). The loss of activity of  $\alpha$ GL $\Delta$ 62-74 is consistent with this hypothesis since the pentamer repeat sequence is absent in the 5'-UR of this mRNA, suggesting an association with the observed translation property of the MHV leader. Thus, it is conceivable, because of its RNA binding properties, that N protein has several functions during infection. It may: (1) act as a *trans*-acting factor to augment translation of the viral mRNAs, as well as (2) play an equally important role in priming transcription of coronavirus RNAs, and (3) serve as a structural protein. In addition to the structural proteins, coronaviruses encode a number of nonstructural proteins whose function(s) is unknown (Lai, 1990). While we favor N protein, these nonstructural proteins have not been ruled out as possible candidate *trans*-acting factors.

A definition of the precise sequences of the *cis*-acting element and the protein(s) which interacts with this sequence will help define the unique role of the 5'-leader RNA in regulating translation. However, the possibility of additional sequence elements which function in translation is suggested by data showing that the 3'-poly(A) tails of certain 3'-UR sequences in cellular and viral mRNAs contain *cis* elements required for translation activity (for review see Jackson, 1993). MHV mRNAs also contain common 3'-UR regions, as a result of the transcription mechanism (Lai, 1990), thus leaving open the possibility that these sequences may also have functional roles in viral protein synthesis.

Overall translation is depressed in MHV-infected cells

(Hilton *et al.*, 1986; this paper, Fig. 2). It is clear from our results that increased activity of MHV mRNAs during infection may be a compensatory mechanism to ensure adequate viral protein synthesis during shutoff of host translation. It was previously thought that specific virus families shut off host cell translation by inactivating certain key initiation factors (Schneider and Shenk, 1987); for example eIF-4F inactivation is the hallmark of picornavirus infection (Sonenberg, 1990). However, recent reports suggest a more complex and universal scenario. Thus eIF-4F inactivation has been reported in adenovirus (Huang and Schneider, 1991) and influenza infections (Feigenblum and Schneider, 1993) where previously it was thought that only eIF-2 was affected (Katze *et al.*, 1986). Similarly eIF-2 phosphorylation occurs during poliovirus infection (Black *et al.*, 1989) in addition to the well-documented inactivation of eIF-4F. These results suggest that different viruses may subject the cell to the same types of possible initiation factor modifications, although the temporal patterns of these modifications may differ among virus types. While earlier MHV studies indicated that translation initiation factors were not modified (Hilton *et al.*, 1986; Baric *et al.*, 1988), recent results suggest that it is likely that selective modifications to initiation factors may contribute to translation shutoff during MHV infection.

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