Evidence for the Presence of an Inhibitor on Ribosomes in Mouse L Cells Infected with Mengovirus

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After infection of mouse L cells with mengovirus, there is a rapid inhibition of protein synthesis, a concurrent disaggregation of polysomes, and an accumulation of 80S ribosomes. These 80S ribosomes could not be chased back into polysomes under an elongation block. The infected-cell 80S-ribosome fraction contained twice as much initiator methionyl-tRNA and mRNA as the analogous fraction from uninfected cells. Since the proportion of 80S ribosomes that were resistant to pronase digestion also increased after infection, these data suggest that the accumulated 80S ribosomes may be in the form of initiation complexes. The specific protein synthetic activity of polysomal ribosomes also decreased with time of infection. However, the transit times in mock-infected and infected cells remained the same. Cell-free translation systems from infected cells reflected the decreased protein synthetic activity of intact cells. The addition of reticulocyte initiation factors to such systems failed to relieve the inhibition. Fractionation of the infected-cell lysate revealed that the ribosomes were the predominant target affected. Washing the infected-cell ribosomes with 0.5 M KCl restored their translational activity. In turn, the salt wash from infected-cell ribosomes inhibited translation in lysates from mock-infected cells. The inhibitor in the ribosomal salt wash was temperature sensitive and micrococcal nuclease resistant. A model is proposed wherein viral infection activates (or induces the synthesis of) an inhibitor that binds to ribosomes and stops translation after the formation of the 80S-ribosome initiation complex but before elongation. The presence of such an inhibitor on ribosomes could prevent them from being remobilized into polysomes in the presence of an inhibitor of polypeptide elongation.

The inhibition of host protein synthesis by picornaviruses has been the subject of numerous investigations. Of the picornaviruses, poliovirus, encephalomyocarditis virus, and mengovirus have been studied most frequently in this regard. In poliovirus-infected HeLa cells, the initiation of cellular capped mRNA translation is impaired (16, 31). Recently, several groups have found a correlation between the degradation of the 220,000-dalton subunit of eucaryotic initiation factor eIF-4F, which is needed for the translation of capped mRNAs (12), and the appearance of inhibition (10). It has also been shown that lysates from poliovirus-infected cells are limiting in this cap-binding protein or cap recognition factor (9).

Encephalomyocarditis virus uses a different strategy to regulate the protein synthesis machinery of cells. In Krebs ascites tumor cells infected with this virus, there is little or no overall decline in the rate of total protein synthesis until relatively late in infection (20, 21). In this case, competition at the mRNA level seems to play a fundamental role in the switchover from host to viral protein synthesis (19).

Much less is understood about the mechanism of inhibition of host protein synthesis by mengovirus, a cardiovirus in the picornavirus family. Mengovirus infection of mouse L cells results in a rapid inhibition of host protein synthesis (5, 8). During this time there is a disaggregation of polysomes and an increase in 80S ribosomes (5, 8). It is clear from studies with mengovirus-infected L cells and Ehrlich ascites tumor cells that there is no inactivation or degradation of host mRNA, since after virus infection, the size distribution (5) and coding capacity of host mRNA in cell-free protein synthesis are preserved (4, 13). Most of the polyadenylated mRNA remains bound to ribosomes throughout the course of infection (4, 5). During early times after infection, the mRNA remains associated with polysomal ribosomes, but then later shifts to the monomosomal region (4, 11). In Ehrlich ascites tumor cells, an enhanced phosphorylation of ribosomal protein S6 and other cytoplasmic proteins has been reported after mengovirus infection (33).

Using fractionated cell-free translation systems from Ehrlich ascites tumor cells to determine what alterations occur in the translational machinery of the host cell following mengovirus infection, Hackett et al. (15) found a 30 to 40% reduction of translation in the virus system relative to the mock system. They attributed the decreased activity to a lower rate of polypeptide chain elongation (15). The pH 5 enzyme fraction was responsible for the decrease in protein synthesis, and not the initiation factor fraction or ribosomal subunits (15).

The results reported herein represent a continuation of our studies concerning the problem of how mengovirus takes over the protein synthesis machinery of the host cell after infection. The focus of this report is on the characterization of the 80S ribosomes that accumulate after infection. The results demonstrate that in intact, infected cells, the 80S ribosomes that accumulate cannot be recruited into polysomes under an elongation block. Most of these 80S ribosomes are resistant to degradation by pronase, suggesting that they are bound to mRNA. Since the 80S-ribosome fraction also contains initiator Met-tRNA, it is suggested that the accumulated 80S ribosomes may represent blocked initiation complexes.

Using cell lysates as a means of attempting to understand better the nature of the lesion in the translational machinery of infected cells, we have found that an inhibitor which binds

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to ribosomes is present in infected cells. The inhibitor is heat labile and can be removed from ribosomes by 0.5 M KCl. A model to explain its action is presented. An additional conclusion from these studies is that there is no uniform strategy used by picornaviruses as a group to interfere with host protein synthesis.

MATERIALS AND METHODS

Buffers. Phosphate-buffered saline (PBS) contained 10 mM sodium phosphate buffer (pH 7.5) and 137 mM NaCl. Hypotonic buffer contained 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.4), 1.5 mM magnesium acetate, 15 mM KCl, and 1 mM dithiothreitol (DTT). mRSB buffer contained 2 mM magnesium acetate, 150 mM KCl, and 10 mM HEPES-KOH (pH 7.4). HKMD buffer (10×) contained 300 mM HEPES-KOH (pH 7.4), 1.2 M KCl, 50 mM magnesium acetate, and 20 mM DTT.

Cells and culture conditions. Mouse L cells were grown either in suspension culture or in cell culture dishes. For suspension culture conditions, the cells were maintained in exponential growth at densities ranging from 3 × 10⁵ to 9 × 10⁵ cells per ml by dilution in Eagle minimal essential medium supplemented with serine, asparagine, and proline in 10 times the amount normally present in minimal essential medium plus calf and fetal bovine serum (5% final concentration of each). Cells were grown at 37°C in Spinner flasks. Confluent monolayers of L cells were grown in plastic Falcon cell culture dishes with diameters of either 60 or 100 mm. Cells were maintained in Richter minimal essential medium supplemented with 5% calf serum.

Virus growth and purification. Preparation and purification of mengovirus were done essentially as described by Abreu and Lucas-Lenard (1). A primarily large-plaque variant (6) stock of mengovirus was used throughout these studies. Titers ranged from 10¹⁰ to 10¹⁵ PFU/ml.

Preparation of cell-free protein synthesis system. Approximately 2.0 liters of exponentially growing L cells in suspension culture were harvested by centrifugation at 700 × g for 3 min. After being washed one time with PBS prewarmed to 37°C, the cells were suspended at a density of 2 × 10⁶ cells per ml in medium without serum and divided into two aliquots. One aliquot was mock infected with medium, and the other was infected with mengovirus at a multiplicity of infection (MOI) of 25 PFU per cell. Adsorption was for 45 min at 37°C with slow stirring (3). After adsorption, the cell density was restored to 5 × 10⁹ cells per ml by dilution with fresh medium containing 5% calf serum and 5% fetal bovine serum. Cells were incubated at 37°C for 3 to 3.5 h and then harvested by centrifugation at 700 × g for 3 min. After being washed three times with PBS, the pelleted cells were suspended in 1.5 volumes of ice-cold hypotonic buffer, allowed to swell for 8 min on ice, and lysed with 20 strokes of a Dounce homogenizer. Cell lysates were then centrifuged at 14,500 × g for 15 min at 4°C in a Sorvall SS34 rotor. To the postmitochondrial supernatant fractions, or S-15s, was then added 1/10 volume of 10× HKMD stock solution. The lysates were either divided into aliquots and stored at −70°C or processed further by ultracentrifugation as described below.

Cell fractionation. Ribosomes (40S subunits and greater) were isolated from the S-15s by centrifugation at 160,000 × g for 2.5 h at 4°C. The resulting ribosome pellet was first rinsed with hypotonic buffer and then suspended in the following salt-washing buffer for 30 min at 4°C: 500 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 0.1 mM EDTA, 250 mM sucrose, 5 mM HEPES-KOH (pH 7.4). The ribosome-free supernatant (S-100) was stored at −70°C. The salt-wash fraction was separated from the ribosomes by centrifuging the resuspended ribosome pellet at 160,000 × g for 2.5 h at 4°C and collecting the supernatant. The supernatant fraction (ribosomal salt wash [RSW]) was then dialyzed for 4 to 5 h against a 0.4 M KCl, 1.2 mM magnesium acetate, 1 mM DTT, 0.1 mM EDTA, 10 mM HEPES-KOH (pH 7.4), and 10% glycerol. The salt-washed ribosomal pellet was suspended in hypotonic buffer containing 10% glycerol and then stored in 50-μl aliquots at −70°C.

Cell-free protein synthesis. Except when otherwise indicated, the reaction mixtures contained in a total volume of 25 μl the following components: 20 mM HEPES-KOH (pH 7.4), 80 mM KCl, 3 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, 80 μg of creatine kinase per ml, 1 mM DTT, 1 to 5 μCi of [35S]methionine (specific activity, approx. 1,000 Ci/mmol), 19 complementary amino acids each at a concentration of 100 μM, 13 μM methionine, an optimal amount of S-15s (approx. 30 to 60 μg of protein), and mRNA as indicated. Lysates were preincubated with micrococcal nuclease to eliminate endogenous template activity as previously described (28). Rabbit reticulocyte initiation factors were donated by Albert Wahba (University of Mississippi Medical Center). Their purification is described in detail elsewhere (7).

The incubation mixture for protein synthesis in fractionated cell extracts contained 25 mM HEPES-KOH (pH 7.4), 80 mM KCl, 3 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, 80 μg of creatine kinase per ml, 1 mM DTT, 1 to 5 μCi of [35S]methionine (specific radioactivity, same as above), each of the naturally occurring amino acids except methionine at a concentration of 100 μM, 10 μM methionine, 1 to 2 μg of rabbit liver RNA (GIBCO Laboratories), 15 to 30 μg of crude RSW fraction, 15 to 20 μg of S-100, and 0.05 to 0.20 A₂₆₀ units of salt-washed ribosomes in a volume of 25 μl.

All reaction mixtures were incubated at 31°C for 30 min (unless otherwise indicated) and analyzed for radioactivity incorporated into protein by precipitation with 5% hot trichloroacetic acid as described previously (18).

Polyosome analysis. Exponentially growing L cells (50 to 100 ml) were mock infected or infected with mengovirus and harvested by centrifugation as described in the previous section. The pellets were washed twice in ice-cold PBS containing 50 μg of cycloheximide per ml (final concentration) to prevent further polypeptide chain elongation. The supernatant was removed, and the cell pellets were lysed by the addition of mRSB buffer containing 0.4% Triton X-100 and 50 μg of cycloheximide per ml. Samples were gently vortexed, and the cell debris was removed by centrifugation in a microcentrifuge for 3 min. The resultant supernatant fractions were layered over 13.2-ml 15 to 40% sucrose-mRSB buffer gradients containing 50 μg of cycloheximide per ml and then centrifuged at 35,000 rpm for 120 min at 4°C in a Beckman SW40 rotor. The gradients were collected and analyzed by standard procedures.

To determine if the virus-induced disaggregation of polysomes could be partially reversed, polysome analysis was performed as described above except that at 3.25 h postinfection, mock- and mengovirus-infected L-cell cultures were each divided into two aliquots. Cycloheximide was added to one aliquot of each culture at a final concentration of 2 μg/ml for 0.5 h. At 3.75 h, all cell cultures were
collected by centrifugation, and the polysomes were analyzed.

Transit time determinations. Measurements of polypeptide chain elongation and termination rates were done as described by Jaye et al. (18).

Measurement of protein synthesis activity of infected-cell polysomes. The relative protein synthesis activity of infected-cell polysomes as a function of time postinfection was determined by the procedure described by Silverstein and Engelhardt (35). Briefly, this determination involved dividing the ratio of the rates of polypeptide synthesis in infected and mock-infected cells by the ratio of the amounts of ribosomes present as polysomes in infected and mock-infected cells. The experiment was performed as follows. At various times postinfection, 100-ml samples of mock-infected and infected cultures were removed and pulse-labeled with [35S]methionine for 15 min (length of time in which incorporation is linear). The latter data were used to determine the relative rates of protein synthesis in mock- and mengovirus-infected cells. The relative amount of ribosomes present as polysomes was determined by weighing cut-out Xerox copies of the polysome tracings.

Assay conditions for measuring the levels of initiator Met-tRNA associated with 80S ribosomes. Sucrose gradient-purified 80S-ribosome fractions from mock-infected and infected cells were collected by centrifugation and extracted with phenol two times. The aqueous phase was adjusted to 0.2 M in ammonium acetate, and 2 volumes of 95% ethanol were added. The RNA was precipitated and collected by centrifugation. The aminoacylated tRNA was deacylated with a mild-base treatment as previously described (25). The enzyme fraction used for the charging of the aforementioned RNA fraction was derived from Escherichia coli by the method of Bose et al. (2). The charging reaction (50 μl) included the following components: 75 mM Tris hydrochloride (pH 7.4), 10 mM magnesium acetate, 10 mM KCl, 2 mM DTT, 4 mM ATP, 1 mM CTP, 20 μM methionine, 0.1 μCi of [35S]methionine per μl, 0.1 μg of crude E. coli synthetases per μl, and various amounts of tRNA. The reaction mixture was incubated at 37°C for 15 min. The level of cold-trichloroacetic acid-precipitable radioactivity in the samples was determined by liquid scintillation counting. Three different amounts of each RNA sample were tested to ensure that subsaturating levels were present.

Susceptibility of polysomes and monosomes to pronase. Ribosome-mRNA associations were assayed by the differential sensitivity of free and mRNA-complexed ribosomes to pronase as previously described (17, 34). Briefly, 1.5 A 260 units of lysate from mock- or mengovirus-infected L cells were incubated at 4°C for 1 h in the presence of 22.5 μg of pronase and 300 U of ribonuclease inhibitor (RNasin, Promega Biotech). Polysomes were then analyzed by sedimentation velocity centrifugation as described above.

RESULTS

Time course of inhibition of protein synthesis by mengovirus. Figure 1 shows a time course of inhibition of protein synthesis in L cells that have been infected with mengovirus at a MOI of 25. It is apparent from these data that the inhibition occurs very rapidly. By 2 h after infection, the rate of protein synthesis is only 50% of that of the uninfected control culture. If the products of this time course are examined by polyacrylamide gel electrophoresis (Fig. 2), it is evident that mengovirus proteins eventually become the predominant proteins synthesized. The change in rate of protein synthesis inhibition after 2 h of infection probably results from the induction of viral protein synthesis.

Polysome-rebuilding experiments. Mengovirus infection of L cells results in a considerable disaggregation of host cell polysomes with a concomitant accumulation of 80S ribosomes (5). As a first attempt to localize possible alterations in the protein synthesis machinery of infected cells, the ability of the accumulated 80S ribosomes to be mobilized into polysomes in the presence of low levels of cycloheximide, an inhibitor of elongation, was examined. Using these conditions, Jaye et al. (18) found that 80S ribosomes that accumulate in vesicular stomatitis virus (VSV)-infected L cells can be mobilized into polysomes. Therefore, VSV-infected L cells provided a positive control for polysome disaggregation reversal and were used in conjunction with polysome-rebuilding experiments conducted with mengovirus-infected cells.

Analysis of the lysate obtained from mock-infected cells by sucrose gradient centrifugation showed that most of the ribosomes sedimented in the polysome region of the gradient, reflecting the high rate of protein synthesis (Fig. 3A). The addition of cycloheximide resulted in a decrease in 80S particles and an increase in polysomes (Fig. 3B). In VSV-infected cells, there was a considerable disaggregation of polysomes and a large accumulation of 80S ribosomes (Fig. 3C), which were readily chased back into polysomes in the presence of cycloheximide (Fig. 3D). Polysomes in cells infected with mengovirus were also considerably disaggregated (Fig. 3E), and there was a large accumulation of 80S particles. However, these 80S particles were unable to be mobilized into polysomes in the presence of cycloheximide, in contrast to the reversal obtained under similar conditions with VSV-infected cells (Fig. 3F). These data suggest that...
inhibited. To address this question, mean ribosome transit times were determined in control and infected L cells. One-half the transit time in uninfected and infected cells was found to be 1.4 min (data not shown). Since the transit time is the time necessary for a ribosome to synthesize a polypeptide chain on an average-sized message, and since the average-sized message in infected cells is essentially unchanged at 3.5 h postinfection (Fig. 2), these results suggest that the rate of polypeptide elongation is unaltered in infected cells.

Protein synthesis activity of infected-cell polysomes. It should be noted that transit time measurements determine the rate of elongation per functioning ribosome. This measurement does not detect any ribosomes in the polysome region that are "stuck" or "frozen." For instance, Jones et al. (22) have observed that VSV infection of RC-60 cells results in a decrease in the rate of polypeptide synthesis without polysome disaggregation. Since no difference in transit times was seen, they explained this observation by proposing the existence of a population of nonfunctioning "frozen" polysomal ribosomes.

Although mengovirus infection results in a disaggregation of polysomes (Fig. 3E), this result does not preclude the possibility that some of the remaining polysomes are inactive. To determine whether such a class of ribosomes exists in our intact, infected cells, it was important to determine if the disaggregation observed corresponds to a proportionate decrease in protein synthesis. Therefore, incorporation of [35S]methionine into protein was measured during 15-min pulses at 2, 4, and 6 h postinfection. These samples were processed as described in Materials and Methods, and the relative rate of protein synthesis per microgram of protein was determined for each sample. Simultaneously with the pulsing, samples of each culture were removed, and the relative amount of ribosomes in polysomes was determined on 15 to 40% sucrose gradients. By computing the ratio of the rates of polypeptide synthesis in infected and mock-infected cells and dividing this ratio by the ratio of the amount of ribosomes present as polysomes in infected and mock-infected cells, the specific activity of infected-cell polysomes in terms of counts per minute per A260 unit was determined.

The protein synthesis activity of infected-cell polysomal ribosomes did decrease, but only after 2 h of infection (Table 1). This finding suggests that early in infection all the ribosomes in polysomes are translating mRNA, but later in

<table>
<thead>
<tr>
<th>Time postinfection (h)</th>
<th>cpm/μg</th>
<th>% Mock*</th>
<th>% Polysomes</th>
<th>% Polysomes*</th>
<th>cpm/polysome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>(A/B)</td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>410</td>
<td>35</td>
<td>57</td>
<td>38</td>
<td>0.91</td>
</tr>
<tr>
<td>Mengo</td>
<td>144</td>
<td></td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>479</td>
<td>31</td>
<td>55</td>
<td>56</td>
<td>0.55</td>
</tr>
<tr>
<td>Mengo</td>
<td>149</td>
<td></td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>530</td>
<td>20</td>
<td>53</td>
<td>32</td>
<td>0.62</td>
</tr>
<tr>
<td>Mengo</td>
<td>108</td>
<td></td>
<td>17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p.i., Postinfection; Mock, mock-infected cells; Mengo, mengovirus-infected cells.
* A 2-ml culture sample was labeled with [35S]methionine for 15 min as described in Materials and Methods.
* Relative rate of protein synthesis in infected cells divided by relative rate in mock-infected cells × 100.
* Percentage of ribosomes as polysomes was determined by weighing cut-out Xerox tracings of each profile.
* Number of ribosomes in polysomes in infected cells relative to number of ribosomes in polysomes in uninfected cells × 100.
* Protein synthesis activity per polysomal ribosome in infected cells.
infection, some of the polysomal ribosomes may not be functional.

The idea of a class of frozen ribosomes was further tested by measuring the incorporation of [3H]leucine into nascent protein chains per A260 unit of polysomal ribosomes at 3.5 h after infection. The specific activity of mock-infected cell polysomes, after subtracting the EDTA-resistant counts per minute, was 38,877 cpm per A260 unit; the specific activity for infected-cell polysomes was 19,679 cpm per A260 unit, or 50% of that of the mock-infected cell polysomes. These data are consistent with the presence of some inactive ribosomes in the polysome region.

Composition of the 80S-ribosome fraction accumulated during infection. The accumulation of 80S ribosomes could result from a lesion in ribosome cycling (36). In this case, the 80S couple would be devoid of mRNA. Alternatively, 80S particles could accumulate if there were a lesion after the formation of the initiator methionyl-tRNA-mRNA-80S ribosome initiation complex but before the formation of the first peptide bond. Under these circumstances, one would expect to find an increase in initiator methionyl-tRNA and mRNA associated with the 80S-ribosome fraction from infected cells.

The amount of initiator tRNA acceptor activity present in the 80S fractions from 3.5-h mock-infected and infected cells was determined as described in Materials and Methods. Approximately 1.8-fold more initiator methionyl-tRNA was present in the infected-cell 80S-ribosome fraction than in the mock-infected cell fraction (data not shown). Furthermore, the total amount of message in the 80S-ribosome fraction from infected cells was twice that in the 80S-ribosome fraction from uninfected cells, as measured by translation of the RNA extracted from these fractions in a wheat germ protein synthesis system (data not shown). While these data do not prove that the additional initiator tRNA and mRNA present in the infected-cell 80S-ribosome fraction are associated with 80S ribosomes, the experiment described below lends support to this possibility.

Susceptibility of the 80S-ribosome fraction from infected cells to digestion by pronase. An 80S-ribosome-mRNA complex can be differentiated from a free 75S-ribosome couple on the basis of its differential sensitivity to treatment with low concentrations of pronase at 4°C (17); 80S-ribosome-mRNA complexes are resistant, whereas free 75S-ribosome couples are cleaved into 40S and 60S ribosomal subunits. Accordingly, extracts from mock-infected and infected L cells were treated with pronase, and then the polysomes were displayed on 15 to 40% sucrose gradients. Absorbance was monitored during fractionation of the gradients, and the proportion of total absorbance in each of the
ribosome-containing fractions was tabulated. The ribosomes within polysomes from either infected or control cells were almost completely resistant to cleavage by pronase (Table 2). This result was expected, since these ribosomes are associated with mRNAs. However, two-thirds of the control and only one-third of the infected 80S ribosomes were cleaved with pronase to yield 40S and 60S ribosomal subunits. These results suggest that the 80S ribosomes that accumulate during infection are in ribosome-mRNA complexes and support the possibility that the extra initiator Met-tRNA and mRNA present in the 80S-ribosome fraction from infected cells may be bound to ribosomes. The accumulation of ribosomes in such 80S-ribosome-mRNA complexes may explain the inability of ribosomes to be "chased" into polysomes in the presence of cycloheximide (Fig. 3).

Protein synthesis in lysates from mock- and mengovirus-infected L cells. To learn more about the mechanism of protein synthesis inhibition by mengovirus, it was thought important to establish a cell-free translation system capable of reproducing the characteristics of the inhibition seen in intact cells. To address this problem, lysates from cells infected for different lengths of time were prepared, and the ability of these lysates to incorporate [35S]methionine relative to that of the uninfected-cell lysate was determined. The inhibition of protein synthesis which is extant as early as 1 h and 30 min after adsorption in intact cells (Fig. 1) was retained in the S-15 lysate prepared from infected cells (data

![Graph](http://jvi.asm.org/)

**FIG. 4.** Translation of exogenous mRNA in nuclease-treated S-15 cell-free translation systems. mRNA as indicated was added to nuclease-treated S-15s. Conditions of nuclease treatment are described in Materials and Methods. Nuclease treatment was for 6 min at 20°C. Symbols: ○, mock-infected S-15; □, mengovirus-infected S-15.

<table>
<thead>
<tr>
<th>TABLE 2. Susceptibility of polysomes and monosomes to pronase</th>
</tr>
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<tbody>
<tr>
<td>Sample(^a)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mock infected</td>
</tr>
<tr>
<td>Mock infected + pronase</td>
</tr>
<tr>
<td>Mengovirus infected(^b)</td>
</tr>
<tr>
<td>Mengovirus infected + pronase</td>
</tr>
</tbody>
</table>

\(^a\) Mock-infected- or infected-cell lysate (1.5 \(A_{340}\) units) was treated with 22.5 \(\mu\)g of pronase for 1 h as described in Materials and Methods.

\(^b\) After treatment, the lysates were subjected to sucrose gradient centrifugation. Tracings of the profiles were cut out, and the fractions of ribosomes as polysomes, 80S ribosomes, and subunits were recorded.

\(^b\) Infection was for 3.5 h.
not shown). This reduced level of translation in the infected-cell lysates was exhibited at all concentrations of Mg\(^{2+}\) and K\(^+\) tested, including those concentrations optimal for protein synthesis (data not shown).

To test the ability of the mock- and mengovirus-infected cell lysates to translate exogenously added mRNAs, each lysate was made mRNA dependent by preincubation with micrococcal nuclease (28). Figure 4 shows the results of typical mRNA titration curves with each of the lysates. Lysates from 3.5-h infected cells were less efficient in translating both viral and host mRNA than were comparable lysates from mock-infected cells, suggesting that the inhibition of protein synthesis exerted by mengovirus in vitro may be nonselective.

**Attempts to restore the translational activity of lysates from infected cells to that of lysates from uninfected cells.** In an attempt to characterize the lesion in infected-cell lysates, partially purified rabbit reticulocyte initiation factors were added to the lysates with the idea that if there was a deficiency in one or more initiation factors, the factor(s) would stimulate protein synthesis in the lysate. Fractions of reticulocyte salt wash containing initiation factors eIF-2, eIF-3, eIF-4A, eIF-4B, eIF-4C, eIF-4D, eIF-4F, eIF-5, and eIF-6 were tested, in addition to elongation factor EF-Tu. These fractions did not stimulate translation in lysates from infected cells to any significant degree (Table 3). Furthermore, neither unprepared preparations of initiation factors (RSW) nor an S-100 fraction from uninfected L cells relieved the inhibition in the infected-cell lysates. The possibility that these lysates contain an active inhibitor which is able to inactivate factors needed for translation upon their addition could not be excluded from this type of experimentation.

**Fractionation of lysates from uninfected and infected cells.** A closer examination of the restriction in translation in

<table>
<thead>
<tr>
<th>Conditions and amount of factor used</th>
<th>cpm + factors(^a)</th>
<th>cpm − factors</th>
<th>Mock infected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-150 phosphocellulose(^a) cut: eIF-4A, eIF-4D (0.37 A(_{260}) units)</td>
<td>0.92</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-450 phosphocellulose cut: eIF-4B, eIF-4F, eIF-3 (9.6 µg)</td>
<td>1.34</td>
<td>0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-450 phosphocellulose cut, followed by sucrose gradient centrifugation: eIF-3 (0.015 A(_{260}) units)</td>
<td>0.97</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Lysates were prepared 3.25 h after infection or mock infection. Results are tabulated as a ratio of the \(^{35}\)S)methionine counts per minute incorporated in the lysates in the presence of the initiation factor fraction divided by the counts per minute incorporated in the absence of the fraction. A ratio of greater than 1 defines stimulation by that fraction; a ratio of less than 1 defines an inhibition by that fraction.

\(^b\) Saturating levels of initiation factors were added to each 25-µl reaction mixture. Factor purification methods were as described by Dratewka-Kos et al. (7).

**FIG. 5.** Translational activity upon reconstitution of the fractionated systems from mock-infected and infected cells. Protein synthesis reactions were performed as described in Materials and Methods. Each 25-µl incubation contained 17 µg of S-100 (mock or mengovirus infected), 30 µg of RSW (mock or mengovirus infected), and 0.04 A\(_{260}\) units of salt-washed (0.5 M KCl) ribosomes (mock or mengovirus infected) as indicated. These amounts were determined to be optimal under the conditions assayed. Incorporation was measured by removing duplicate 4-µl samples after 30 min of incubation at 30°C.

infected-cell lysates was attempted by fractionating the S-15 lysates by ultracentrifugation into three crude fractions as follows: a supernatant fraction (S-100; 100,000 × g), an RSW (0.5 M KCl) fraction, and a salt-washed ribosome fraction (P-100). The ability of each fraction derived from infected cells to support translation was tested by a reconstituted mock-infected-cell fractionated system which was made limiting for that particular fraction. Translational activity was operationally defined on the basis of titration curves for each individual fraction.

Both the RSW and S-100 fractions from mock-infected cells appeared more active in supporting endogenous mRNA translation with mock-infected-cell salt-washed ribosomes than did the corresponding fractions from infected cells (Fig. 5). Interestingly, salt-washed ribosomes from mengovirus-infected cells supported approximately the same level of translation as salt-washed ribosomes from mock-infected cells when supplemented with mock-infected cell RSW and S-100 fractions.

These data were unexpected, since the S-100 and RSW fractions from mock-infected cells did not stimulate translation in the unfractinated mengovirus S-15 lysate (data not shown). In an attempt to explain these results, the translational activity of the infected-cell non-salt-washed ribosomes was compared with that of infected-cell salt-washed ribosomes. The infected-cell non-salt-washed ribosomes were 50% less active than the mock-infected-cell non-salt-washed ribosomes (Fig. 6A). However, salt washing the infected-cell ribosomes with 0.5 M KCl resulted in an increase in translational activity, thereby restoring the activity of the infected-cell ribosomes (Fig. 6B). These results are not a consequence of a salt-induced inactivation of mock-infected-cell ribosomes, since the salt-washed and non-salt-washed ribosomes showed the same translational activity based on their A\(_{260}\) content (data not shown).

**Assay for a translational inhibitor.** One explanation for this
168 and 2.0 μCi of ribosomes equaled infected cells.

If 4). ribosomes with 0.5 RSW. The infected-cell amount into L cell RSW the inhibitor activity associated with this fraction (Table 4). These data lend strong support to the notion of the existence of a translational inhibitor in mengovirus-infected L cells.

To characterize further the nature of this inhibitor, the effects of heat and micrococcal nuclease on the activity of the inhibitor were examined. Preincubation of the infected-cell RSW at 70°C for 5 min before its addition to the fractionated translation system destroyed the inhibitory activity associated with this fraction (Table 4). However, treatment of the RSW with micrococcal nuclease failed to abolish its inhibitory effects. The data from these two experiments suggest that the inhibitor found in the infected-cell RSW may be a protein.

Also of interest is the fact that RSW prepared from cells 4 and 6 h after infection appeared to be more inhibitory than RSW prepared from cells 2 h after infection (Fig. 7). These results demonstrate that the inhibitor accumulates in cells with time of infection. It should also be pointed out that the inhibitor correlated with the decrease in the activity of polysomal ribosomes shown in Table 1.

**Step in protein synthesis affected by salt washing of infected-cell ribosomes.** To gain further information about the step affected by the inhibitor, use was made of the initiation inhibitor vanadate, which allows 80S-ribosome initiation complex formation but prevents the first peptide bond from being formed (30). Vanadate had little effect on translation with infected-cell non-salt-washed ribosomes, suggesting that these ribosomes were not able to initiate new polypeptide chains (Fig. 8). However, when vanadate was added to infected-cell salt-washed ribosomes, translation was reduced to the level of that with non-salt-washed ribosomes. Since the concentrations of vanadate used had no effect on polypeptide elongation (data not shown), these data suggest that the primary effect of removal of the inhibitor by salt washing is an increase in initiation. The extended period of amino acid incorporation with infected-cell salt-washed ribosomes also indicates an increase in initiation capability.

The data presented above strongly suggest the existence of a class of inactive 80S ribosomes. If such a class exists, it is possible that salt treatment was somehow removing some inhibitor from these ribosomes. To test this possibility, lysates from infected cells were fractionated on sucrose gradients into polysome and 80S-ribosome fractions. Half of the polysome fraction and half of the 80S-ribosome fraction were salt washed as described in Materials and Methods. The other half of each fraction was kept in a low salt solution. Fractionated cell-free translation systems were

> **FIG. 6. Comparison of activities of non-salt-washed and salt-washed ribosomes from infected cells.** The reaction conditions were as described in Materials and Methods. Each 25-μl incubation contained 17 μg of mock-infected-cell S-100, 24 μg of mock-infected-cell RSW, and 2.0 μCi of [3H]-amino acid mixture. (a) Mock-infected-cell non-salt-washed ribosomes; (b) Mock-infected-cell salt-washed ribosomes; (c) infected-cell non-salt-washed ribosomes; (d) infected-cell salt-washed ribosomes. 1 μl of ribosomes equaled 0.016 A260 units. Incubation was for 30 min at 31°C. Duplicate 5-μl samples were analyzed for radioactivity.

<table>
<thead>
<tr>
<th>TABLE 4. Characteristics of inhibitory activity in the RSW</th>
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<tbody>
<tr>
<td><strong>RSW added to sample</strong></td>
</tr>
<tr>
<td>No RSW added ..................................................................</td>
</tr>
<tr>
<td>28 μg of mock RSW ..................................................</td>
</tr>
<tr>
<td>28 μg of mock RSW + 20 μg of mock RSW ........................</td>
</tr>
<tr>
<td>28 μg of mock RSW + 20 μg of mengo RSW .......................</td>
</tr>
<tr>
<td>28 μg of mock RSW + 20 μg of nuclease-treated* mock RSW ....</td>
</tr>
<tr>
<td>28 μg of mock RSW + 20 μg of nuclease-treated mengo RSW ....</td>
</tr>
<tr>
<td>28 μg of mock RSW + 20 μg of mock RSW heated at 70°C for 5 min</td>
</tr>
<tr>
<td>28 μg of mock RSW + 20 μg of mengo RSW heated at 70°C for 5 min</td>
</tr>
<tr>
<td>Nuclease-treated mock-infected salt-washed polysomes ..........</td>
</tr>
</tbody>
</table>

* Fractionated cell-free translation system was prepared as described in Materials and Methods. Incubations were for 60 min at 31°C. Mock RSW, RSW from mock-infected cells; mengo RSW, RSW from mengovirus-infected cells.

* Duplicate 5-μl samples were analyzed. This number represents the average.

* The salt wash was treated with 10 μg of micrococcal nuclease per ml in the presence of 1 mM calcium chloride. The reaction was stopped by the addition of EGTA (ethylene glycol-bis [β-aminoethyl] ether)-N, N', N'-tetraacetic acid) to a final concentration of 3 mM. The treated sample was then added to the reaction mixture.
assembled with combinations of each of these fractions. When non-salt-washed infected-cell 80S ribosomes were added to salt-washed polysomes from infected cells, 9,245 cpm of [35S]methionine was incorporated into protein. When salt-washed infected-cell 80S ribosomes were added to these polysomes, 15,005 cpm was incorporated. These results suggest that salt washing has an effect on 80S ribosomes from infected cells.

DISCUSSION

As shown in this report, L-cell protein synthesis inhibition by mengovirus is rapid and severe. Within 3 h after infection, the synthesis of mengovirus proteins can be detected by pulse-labeling infected cells with [35S]methionine and analyzing the products by polyacrylamide gel electrophoresis (Fig. 2). The decrease in rate of protein synthesis inhibition after 2 h probably represents the induction of viral protein synthesis.

Polysome disaggregation occurs within 2 h after adsorption (Table 1), and there is an accumulation of 80S ribosomes, which cannot be mobilized into polysomes under conditions of an elongation block (Fig. 3). This situation is in contrast to the other virus-infected system studied in our laboratory, i.e., VSV-infected L cells, in which the accumulated 80S ribosomes are readily recruited into polysomes (Fig. 3). The lack of recruitment of 80S ribosomes from mengovirus-infected cells does not appear to be the result of a change in the rate of polypeptide elongation, since mean ribosome transit times were the same in mock- and mengovirus-infected cells at the time during infection (3.25 h) at which the polysome-rebuilding experiments were done. A comparison of the effect of pronase treatment on lysates from mock- and virus-infected cells (Table 2) revealed that two-thirds of the infected and only one-third of the mock-infected 80S ribosomes formed were resistant to the action of this enzyme. Usually, 80S ribosomes that are attached to mRNA are resistant to pronase treatment. Indeed, lysates from infected cells contained twice as many 80S ribosomes, initiator Met-tRNA, and mRNA as lysates from mock-infected cells. It is possible that the 80S ribosomes that accumulate after infection are in the form of 80S-ribosome initiation complexes that cannot engage in elongation. It is postulated that the inability of infected-cell ribosomes to be mobilized into polysomes results from the presence of these blocked 80S-ribosome initiation complexes.

The experiments reported here with cell lysates from infected cells also point to some sort of block at the ribosome level. This block could be removed almost totally by exposing the ribosomes to 0.5 M KCl. The RSW fraction from infected-cell ribosomes was inhibitory when added to a cell-free translation system composed entirely of constitu-

FIG. 7. Translational activity of RSW prepared from infected L-cell ribosomes at different times after infection. RSW was added to fractionated mock-infected cell-free translation systems as described in Materials and Methods. Samples (4 μl) were removed from each incubation at the indicated times, and the amount of [35S]methionine incorporated into protein was determined. Symbols: □, mock-infected-cell salt wash; △, 2-h infected-cell salt wash; △, 4-h infected-cell salt wash; ▽, 6-h infected-cell salt wash; ○, no salt wash.

FIG. 8. Effect of ammonium metavanadate on the translational activity of ribosomes from infected L cells. Fractionated cell-free translation systems contained infected-cell salt-washed or non-salt-washed ribosomes as indicated. Optimal amounts of mock-infected-cell RSW and S-100 were used. Vanadate was added at a concentration of 100 μM. At the times indicated, 3-μl samples were removed from each incubation, and the incorporation of [35S]methionine into protein was measured.
ents from mock-infected cells (Table 4). These results suggest that an inhibitor is present on ribosomes from infected cells and that it can be released by salt. Furthermore, the data suggest that when the salt concentration is decreased, the inhibitor in the RSW can rebind to ribosomes. The inhibitor is unlikely to be a mengovirus capsid protein, even though Manak et al. (26) have shown that mengovirus capsid proteins bind to 40S ribosomal subunits, because these proteins still remain bound to the ribosomes after a salt wash (data not shown).

RSW isolated from infected cells at 2 h after infection did not differ substantially from RSW isolated from mock-infected cells in supporting the translation of mock-infected-cell salt-washed ribosomes (Fig. 7). Also, ribosomes isolated from cells 2 h after infection were not very different in translation activity from those of mock-infected cells, even though total protein synthesis at this time in infection was 50% of the level of mock-infected cells. These data suggest that the inhibitor that is present on ribosomes at 2 h after infection may not be present in sufficient quantity to detect by our rather crude cell-free translation assay. A certain amount must accumulate in cells before it can be measured in these assays.

These results are consistent with the following model of mengovirus-directed protein synthesis inhibition. The virus activates (or induces the synthesis of) a temperature-sensitive, nuclease-resistant (Table 4) inhibitor of translation that binds to ribosomes and acts either directly or indirectly by modifying some ribosomal component. (It is not clear whether the inhibitor is of viral or cellular origin.) The net result of the inhibition is an accumulation of 80S-ribosome initiation complexes that cannot engage in elongation and thus cannot enter polysomes in the presence of cycloheximide. These complexes may be responsible for the pronase resistance of 80S ribosomes. High concentrations of salt can release the inhibitor, enabling elongation, initiation, or both to occur.

To explain the gradual decrease in polysomal-ribosome activity, it is predicted that the inhibited 80S ribosomes are able to migrate along the message without actually synthesizing protein. This possibility has also been suggested to account for the restoration of viral protein synthesis in VSV-infected rabbit cornea cells (22). Those ribosomes that do not have inhibitor attached to them will continue to elongate at the same rate, thereby providing an explanation for the lack of difference in transit times in mock-infected and infected cells.

Mengovirus-infected cell lysates do not possess an enzymatic activity capable of degrading the 220,000-dalton subunit of initiation factor eIF-4F (27) which is cleaved in HeLa cells infected with poliovirus. Therefore, the mechanisms by which mengovirus and poliovirus inhibit cellular protein synthesis are different. Also, since mengovirus infection results in the production of an inhibitor, the mechanism by which this virus shuts off host protein synthesis differs from the mechanism by which encephalomyocarditis virus accomplishes this phenomenon.

Although overall protein synthesis appears to be inhibited in mengovirus-infected cells, the high efficiency with which mengovirus RNA is translated (at least in vitro) probably compensates for this decrease. Indeed, it has been shown (1, 14, 23, 32) that on a molar basis, mengovirus mRNA competes 35 times more effectively in translation than does globin mRNA; that is, half-maximal inhibition of globin synthesis occurred in lysates that contained 35 molecules of globin mRNA for every molecule of mengovirus RNA. Also, mengovirus mRNA binds eIF-2 with a 30-fold-higher affinity than globin mRNA (29, 32). These observations suggest that under limiting conditions of protein synthesis, mengovirus mRNA may still be translated to a sufficient degree for progeny virus formation.

Experiments are under way in our laboratory to characterize the inhibitor further.

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LITERATURE CITED


