Cellular Synthesis and Modification of Murine Hepatitis Virus Polypeptides

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SUMMARY

Mouse L fibroblasts infected with mouse hepatitis virus, MHV3, and radiolabelled with $^{35}$S-methionine, contained, in addition to the three major structural polypeptides, p24, p56 and p180, two additional ones, p22 and p50. Of these total five polypeptides, only three, p22, p56 and p180, were labelled in infected cells during a 2 min $^{35}$S-methionine pulse and are, therefore, presumed to be immediate translation products. Pulse–chase and chymotryptic peptide mapping experiments showed apparent precursor–product relationships between p56 and p50 and between p22 and p24. Protein synthesis in infected cells was synchronized at the initiation stage by pre-exposure to hypertonic medium. Using a 0·5 min pulse–10 min chase sequence, to limit incorporation of $^{35}$S-methionine to stretches of approx. 100 amino acids adjacent to translational initiation sites, it was found that all three polypeptides, p22, p56 and p180 contained radiolabel. It is thus apparent that translation of the three major structural proteins (or precursors) is initiated independently rather than at a single site as in the cases of other positive-strand RNA viruses such as polio or Semliki Forest virus.

INTRODUCTION

Murine hepatitis viruses (MHV) are pleomorphic, lipid-enveloped virions of about 100 nm diam. The genome is a single-stranded RNA of positive sense which has a mol. wt. of approx. 5·4 x 10$^6$ and is reported to be polyadenylated (Lai & Stohlman, 1978; Wege et al., 1978). Three major size classes of virion polypeptides have been described, i.e. a large glycoprotein of about 180 000 mol. wt., a basic 50 000 to 60 000 mol. wt. nucleocapsid protein and one or two smaller polypeptides of mol. wt. between 20 000 and 25 000 (Anderson et al., 1979; Bond et al., 1979; Sturman, 1977; Stohlman & Lai, 1979; Wege et al., 1979).

Considerable interest has recently focused on a number of MHV strains, largely as a result of their demonstrated ability to undergo persistent infections both in vivo and in vitro (Lucas et al., 1977, 1978; Sorensen et al., 1980) as well as for their potential use as animal virus models relevant for the study of slowly degenerative neuropathies (Weiner, 1973; Weiner et al., 1973; Sorensen et al., 1980). Nevertheless, very little information is available at present regarding the biochemical events which accompany MHV infection and replication within the host cell. As a prelude, therefore, to understanding the mechanisms of cell (tissue)–virus interactions in MHV infections, we have investigated the intracellular synthesis and post-translational modification(s) of virus-coded polypeptides in cultures of mouse L-2 (Rothfels et al., 1959) fibroblasts productively infected with MHV.

METHODS

Cells and virus. The derivation of mouse L-2 fibroblasts, the MHV3 strain of MHV and a description of the plaque assay used for virus titration have been reported previously (Lucas et al., 1977).
Chemicals and radioisotopes. Tunicamycin, originally provided by Dr G. Tamura of the University of Tokyo, was a generous gift of Drs S. Dales and H. Shida of our Department. L-35S-methionine (524 Ci/mmol) was purchased from New England Nuclear.

Infection and labelling conditions. Confluent monolayer cultures of L-2 cells in 60 mm Petri dishes were inoculated at a multiplicity of infection (m.o.i.) of 5 with MHV3. After 60 min adsorption at 4 °C 5 ml minimal essential medium (MEM) supplemented with 5% foetal calf serum (FCS) were added and incubation was continued at 37 °C. Isotopic labelling of synthesized polypeptides was performed by replacing the culture medium with 1 ml methionine-free MEM containing 35S-methionine (10 µCi/ml). For pulse–chase studies, labelling medium used for the pulse was removed by aspiration and the cell monolayer washed once with MEM before incubation in 5 ml MEM supplemented with 5% FCS for the chase period. Glycosylation inhibition was performed by incubating cell monolayers in MEM supplemented with 5% FCS and containing tunicamycin (0, 0.1, 1 or 10 µg/ml) immediately following adsorption. At 6-5 h post-inoculation (p.i.) medium was removed and the cells labelled for 30 min with 1 ml methionine-free MEM containing 35S-methionine (10 µCi/ml) and the appropriate concentration of tunicamycin. Cells were harvested by scraping, spun into pellets at 650 g for 10 min and washed once with phosphate-buffered saline (PBS). For polyacrylamide gel electrophoresis (PAGE) of labelled cell extracts, cell pellets were thoroughly mixed with 0.2 ml 10 mM-tris-HCl pH 7.4 containing 2 mM-MgC12 and 5 µg deoxyribonuclease I (Worthington). After standing for 15 min at 4 °C, 0.2 ml dissociation buffer (7 mM-tris-phosphate pH 6.8, 3.46 M-mercaptoethanol, 30% glycerol, 6% SDS and 0.006% bromophenol blue) were added and mixed.

Virus purification. Glass bottles containing confluent monolayers of L-2 cells (about 2 × 10^7 cells/bottle) were inoculated at an m.o.i. of 0.1 with MHV3 and incubated for 24 h in MEM supplemented with 5% FCS and containing 32S-methionine (1 µCi/ml). Supernatant medium was clarified of cell debris by centrifugation for 15 min at 3000 g. A crude virus pellet was then obtained by centrifugation of the clarified supernatant for 60 min at 23000 rev/min in a Beckman SW27 rotor. The virus pellet was homogeneously resuspended in several ml TN buffer (10 mM-tris–HCl, 100 mM-NaCl pH 7.4) and layered on to a continuous 10 to 45% sucrose gradient. The gradient was centrifuged for 90 min at 22000 rev/min and fractions collected by gravity after piercing the bottom of the tube. Infectious virus, recovered as a band near the middle of the gradient, was diluted with TN buffer and layered on to a second 10 to 45% sucrose gradient. Virus recovered after centrifugation of this second gradient was diluted with 1 vol. TN buffer and pelleted by centrifugation for 60 min at 45000 rev/min in a Beckman SW56 rotor.

Sucrose gradient sedimentation analysis of polyribosomal RNA. For the analysis of polyribosomal RNA, 100 mm culture dishes of MHV3-infected L-2 cells were harvested at 6-7 h p.i. immediately after exposure to hypertonic medium (335 mM-NaCl) for 0, 10 or 40 min. Post-nuclear cytoplasmic extracts were prepared according to Huang & Baltimore (1970), made 1% in sodium deoxycholate and Brij-58 and overlaid on to linear gradients of 15 to 30% sucrose. After centrifugation for 3 h at 24000 rev/min in a Beckman SW27 rotor, tubes were pierced and fractions collected by gravity. The absorbance of each fraction was measured at 260 nm.

Synchronization of protein synthesis by exposure to high-salt medium. Monolayer cultures of MHV3-infected L cells in 60 mm Petri dishes were exposed at 6 h p.i. to hypertonic medium (MEM supplemented with 5% FCS and containing a final NaCl concentration of 335 mM). After 40 min incubation at 37 °C, hypertonic medium was replaced with isotonic MEM containing 32S-methionine (100 µCi/ml). Subsequent pulse labelling for periods of 0.5 to 8 min was terminated by the addition of ice-cold dissociating buffer. For cultures subjected to a pulse–chase sequence, monolayers were washed immediately post-pulse with chase
medium (MEM supplemented with 5% FCS and 1 mM-methionine) and then incubated at 37 °C for 10 min in chase medium.

**Polyacrylamide gel electrophoresis.** SDS-PAGE (Laemmli, 1970) of isotopically labelled cell extracts and of known mol. wt. standard proteins was performed on linear gradients of 5 to 18% acrylamide. After staining and destaining for visualization of protein standard markers, gels were fluorographed (Bonner & Laskey, 1974) using Kodak X-Omat R film. For the quantification of $^{35}$S-methionine in virus polypeptides, dried gel slices were incubated for 12 h in 1 ml Protosol (New England Nuclear), mixed with 8 ml toluene-based scintillant and quantified by scintillation spectrometry.

**Peptide mapping.** $^{35}$S-methionine-labelled virus polypeptides were visualized by autoradiography of dried SDS gels. Corresponding gel slices were washed overnight in methanol to remove SDS and salts, dried under nitrogen and digested for 16 h at 37 °C with 3 ml 0.05 M-ammonium bicarbonate containing 25 μg/ml chymotrypsin (Worthington). Eluted peptides were freed from gel fragments by filtration through glass wool, lyophilized and oxidized with performic acid (Lamb et al., 1978). The oxidized peptides were again lyophilized, clarified by centrifugation and applied to 20 x 20 cm cellulose (Cel 300) plates. Development consisted of electrophoresis in the first dimension for 140 min at 20 mA in acetic acid : pyridine : water (3:3:94, by vol., pH 4.8) and chromatography in the second dimension in butanol : acetic acid : pyridine : water (7:1:5:4, by vol.). $^{35}$S-methionine-labelled peptides were detected by autoradiography.

**RESULTS**

**MHV3 virion polypeptides**

Purified virions of MHV3 were found to contain three major polypeptides, designated p180, p56 and p24, and varying amounts of a minor polypeptide, p22, were occasionally detected. Virion polypeptides of similar mol. wt. have been described for related coronaviruses (Sturman, 1977; Bond et al., 1979; Stohlman & Lai, 1979; Wege et al., 1979) and it thus appears likely that a high degree of structural and genetic similarity exists between the various reported strains of murine hepatitis virus.

**Initial synthesis of three major virus polypeptides in MHV3-infected cells**

Virus-infected cultures of L-2 cells pulsed for 2 min with $^{35}$S-methionine were found to contain three major labelled polypeptides, p22, p56 and p180 (Fig. 1). Over a subsequent chase period the autoradiographic intensity of p22 decreased, apparently in favour of a fourth polypeptide, p24. One explanation for this observation is that p24, unlike p22, p56 and p180, is not a primary translation product but rather is derived post-translationally from p22.

Comparison of the chymotryptic peptide maps of $^{35}$S-methionine-labelled p22 and p24 (Fig. 2) in fact demonstrates strong peptide relatedness; differences between these two polypeptides amount to three extra peptides present in p24 but not in p22, and one extra peptide present in p22 but not in p24 (arrows in Fig. 2). These results are consistent with a precursor-product relationship between p22 and p24 although the type of post-translational modification involved remains uncertain.

Since a commonly encountered mechanism of post-translational viral protein modification is dolichol-mediated glycosylation, we investigated the possibility that p22 might be converted to p24 by such a process. Cultures of MHV3-infected cells were labelled with $^{35}$S-methionine in the presence or absence of tunicamycin, a specific inhibitor of dolichol-mediated glycosylation (Takatsuki et al., 1975). As shown in Fig. 3, the presence of tunicamycin (1 μg/ml) resulted in a shift in apparent mol. wt. of the major viral glycoprotein, p180, but had no effect on the electrophoretic mobilities of any of the other viral proteins, including p24. It
is thus clear that p24 is derived from p22 by a process other than dolichol-mediated glycosylation.

Proteolysis of nucleocapsid protein p56

As previously reported (Anderson et al., 1979), MHV3-infected L-2 cells, which are pulsed with $^{35}$S-methionine and chased for 2 h at 6·5 h p.i., contain a fifth virus polypeptide, p50. It was previously suggested, on the basis of the enriched arginine contents of p56 and p50, that the latter was derived from the former by an intracellular processing event (Anderson et al., 1979).

Conclusive demonstration of the precursor–product relationship between p56 and p50 was provided by chymotryptic peptide mapping (Fig. 2). Although the $^{35}$S-methionine-labelled peptides derived from p50 showed insignificant similarity with those obtained from p180, p24 or p22, they were virtually identical with those obtained from p56.

Hypertonic-induced synchronization of viral protein synthesis

Exposure of cultured cells to hypertonic medium results in cessation of the initiation stage of protein synthesis but allows elongation of nascent peptide chains to proceed (Saborio et al., 1974; Clegg, 1975). After a suitable ‘run-off’ period of about 40 min in which elongating peptides are completed, protein synthesis may be restored in a synchronous manner by re-establishing isotonic conditions. Since the resultant synchrony of protein synthesis is a consequence of simultaneous initiation, a kinetic analysis of the appearance of newly synthesized polypeptides may provide information as to whether such polypeptides are initiated independently or from a common site.

It has been demonstrated in other systems (e.g. Saborio et al., 1974) that exposure of cells to hypertonic medium allows ‘run-off’ of ribosomes bound to mRNA, i.e. resulting in a decrease in cellular polyribosomes. To verify this phenomenon in our system, MHV3-infected cultures were harvested at 6·7 h p.i. immediately after exposure to 335 mM-NaCl for 0, 10 or
Fig. 2. Chymotryptic peptide mapping of MHV3 polypeptides. $^{35}$S-methionine-labelled infected cell extracts were resolved on SDS–PAGE and excised virus polypeptides subjected to chymotrypsin digestion as described in Methods. The digested samples were resolved by two-dimensional mapping, involving electrophoresis from left (anode) to right (cathode) and chromatography from bottom to top. $^{35}$S-methionine-labelled peptides were detected by autoradiography. (a) p22, (b) p24, (c) p50, (d) p56, (e) p180. Arrows in (a) and (b) indicate non-common peptides.
40 min. Analysis of cytoplasmic extracts by sucrose gradient sedimentation in fact demonstrated a progressive disappearance of polyribosomal RNA after high-salt treatment. As shown in Fig. 4 cultures maintained in isotonic medium contained a population of polyribosomal RNA which sedimented between fractions 6 and 10. The amount of polyribosomal RNA was substantially decreased in cells exposed for 10 min to 335 mM-NaCl and was virtually absent in cells exposed to the high-salt treatment for 40 min.

To confirm that exposure of cells to hypertonic medium permits translational elongation but not initiation, MHV3-infected cells were pulse-labelled with 35S-methionine at various times during the 'run-off' period. As shown in Fig. 5, translation of the largest viral glycoprotein, p180, could be observed even after 15 min exposure to hypertonic medium. In contrast, hypertonic exposure inhibited translation of p56 and p22 after little more than 10 and 5 min respectively. These results confirm that protein translation is inhibited fairly rapidly after high-salt exposure. Moreover, they strongly suggest that elongation of a polypeptide
Synthesis of MHV polypeptides encoded by a long mRNA molecule (e.g. of the size required to encode p180) may continue for some time after translational initiation has been blocked.

Incidentally, it should be noted that during high-salt exposure, a presumed cell protein (designated X in Fig. 5) was observed. Enhanced synthesis of cell proteins has also been demonstrated in other cell lines as a result of virus infection and/or hypertonic exposure (e.g. Peluso et al., 1977). Their significance is as yet unknown.

In the experiments designed to investigate synchronous protein synthesis in MHV3-infected cells, monolayer cultures were labelled with $^{35}$S-methionine at 6·7 h.p.i., at which time host cell protein synthesis is strongly inhibited. To confirm synchrony of protein synthesis, MHV3-infected L cell monolayers, exposed from 6 to 6·7 h.p.i. to 335 mM-NaCl, were labelled immediately afterwards with $^{35}$S-methionine in isotonic medium for pulses of 0·5, 1, 2, 4, 6 and 8 min. As shown in Table 1 the approximate pulse times required for the appearance of appreciably labelled p22, p56 and p180 are 1 min, 2 to 4 min and 6 to 8 min respectively. Thus, the chronological order of appearance of labelled polypeptides reflects the order of increasing mol. wt. (and of increasing time required for complete translation). Using non-synchronous conditions, i.e. without prior exposure to hypertonic medium, all three virus polypeptides were labelled with $^{35}$S-methionine, even at the shortest (0·5 min) pulse (data not shown). Since reported rates of translation are of the order of 28,000 mol. wt. (or 260 amino acids) protein/min (Clegg, 1975), the expected rates of complete synthesis of p22, p56 and p180 are 0·8, 2·0 and 6·4 min respectively. Thus, it is apparent that the observed rates of appearance of labelled virus polypeptides can only be explained by assuming synchronous translational initiation.

To examine the mode of translational initiation of virus polypeptides, synchronized cells

Fig. 5. Polypeptides synthesized in MHV3-infected cells after exposure to 335 mM-NaCl for (a) 0, (b) 5, (c) 10, (d) 15 and (e) 20 min. At the times indicated, cells were labelled for 5 min with $^{35}$S-methionine and subsequently harvested.
<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>0.5 min pulse</th>
<th>1 min pulse</th>
<th>2 min pulse</th>
<th>4 min pulse</th>
<th>6 min pulse</th>
<th>8 min pulse</th>
<th>10 min chase</th>
</tr>
</thead>
<tbody>
<tr>
<td>p22</td>
<td>&lt;20 (−)</td>
<td>429 (74)</td>
<td>937 (47)</td>
<td>2023 (43)</td>
<td>3257 (36)</td>
<td>4241 (33)</td>
<td>305 (29)</td>
</tr>
<tr>
<td>p24</td>
<td>&lt;20 (−)</td>
<td>50 (9)</td>
<td>120 (6)</td>
<td>296 (6)</td>
<td>823 (9)</td>
<td>1593 (12)</td>
<td>137 (13)</td>
</tr>
<tr>
<td>p56</td>
<td>&lt;20 (−)</td>
<td>78 (13)</td>
<td>905 (45)</td>
<td>2317 (49)</td>
<td>4519 (49)</td>
<td>5176 (41)</td>
<td>482 (45)</td>
</tr>
<tr>
<td>p180</td>
<td>&lt;20 (−)</td>
<td>23 (4)</td>
<td>41 (2)</td>
<td>98 (2)</td>
<td>549 (6)</td>
<td>1749 (14)</td>
<td>140 (13)</td>
</tr>
<tr>
<td>Total</td>
<td>− (−)</td>
<td>580 (100)</td>
<td>2003 (100)</td>
<td>4734 (100)</td>
<td>9148 (100)</td>
<td>12759 (100)</td>
<td>1064 (100)</td>
</tr>
</tbody>
</table>

Table 1. $^{35}$S-methionine labelling of virus polypeptides after hypertonic synchronization of host cell protein synthesis
were subjected to a short pulse–chase sequence in order to specifically label peptide regions adjacent to translational initiation sites. A 0.5 min $^{35}$S-methionine pulse was chosen since this labelling period should allow initiation and synthesis of a peptide sequence of appreciable length (of the order of 100 amino acids) but would not permit complete synthesis of even the shortest (p22) virus polypeptide (compare Table 1). A subsequent chase of 10 min was performed to permit completion of polypeptides which had been labelled during the pulse. As shown in Table 1 all three virus polypeptides, p180, p56 and p22 (including its post-translational product, p24) were found to be radiolabelled by this pulse–chase procedure. Taken together, the results of Table 1 strongly suggest independent translational initiation of these three virus polypeptides.

**Discussion**

The results of the present study demonstrate the presence in MHV3-infected cells of three major, independently initiated virus polypeptides. Two of these polypeptides, p180 and p56, were incorporated into progeny virions; the third, p22, was largely post-translationally modified to p24 before inclusion into virions.

The post-translational process which transforms p22 to p24 remains unclear. A common mechanism of virus polypeptide modification, such as that involving dolichol-mediated glycosylation is evidently not involved. Previous reports have suggested the presence of an MHV glycoprotein within the mol. wt. range of 23 000 to 25 000 (Sturman & Holmes, 1977; Wege et al., 1979). However, generally much lower amounts of radiolabelled glucosamine were found to be incorporated into this low mol. wt. protein as compared to the major glycoprotein, p180. Isotopically labelled fucose is not detected in the smaller protein (Sturman & Holmes, 1977). Since radiolabelled glucosamine may undergo metabolic alteration resulting in integration of radioactivity into non-carbohydrate-containing molecules (e.g. Krystal et al., 1976), the precise nature of p24 must await more rigorous investigation. In compliance with the data available at present, p24 may contain low amounts of carbohydrate which are added post-translationally to p22 by a mechanism other than the lipid-linked glycosylation pathway (Parodi & Leloir, 1979) commonly observed in virus glycoprotein biosynthesis.

Cleavage of viral p56 to p50 in MHV3-infected cells may be analogous to reported proteolytic reactions on nucleocapsid 'N' proteins in cells infected with various paramyxoviruses (Mountcastle et al., 1970, 1974; Mountcastle & Choppin, 1977). It has been suggested in these latter cases that virus assembly may be inhibited as a result of nucleocapsid protein processing (Mountcastle et al., 1970). In this regard it is conceivable that proteolysis of nucleocapsid protein, p56, in MHV3-infected L cells may interfere with virion assembly and thereby account for the generally lower yields of progeny MHV obtainable from this (Lucas et al., 1978) as compared with other cell lines (Sturman et al., 1980; Siddell et al., 1980).

It is evident from the results presented that coronaviruses, as exemplified by MHV3, differ in protein translational strategy from other well-characterized positive-strand RNA viruses such as enteroviruses (e.g. polio) or alphaviruses (e.g. Semliki Forest virus). In these latter cases, viral structural proteins are translated sequentially from a viral messenger RNA containing a single initiation site (Jacobson & Baltimore, 1968; Clegg & Kennedy, 1975). The present study demonstrates independent translational initiation of the three major structural polypeptides of MHV3. A similar phenomenon of independent initiation of viral protein synthesis has been demonstrated for the flavivirus, Kunjin (Westaway, 1977). It remains to be determined as to whether independent translational initiation of MHV3 polypeptides is a reflection of multiple initiation sites on a polycistronic mRNA or alternatively of single initiation sites on separate mRNAs. Studies with two other
coronaviruses, the JHM strain of MHV (Siddell et al., 1980) and avian infectious bronchitis virus (Stern & Kennedy, 1980) suggests that coronavirus replication may in fact involve separate mRNA species.

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Note added in proof: Our recent experiments (Cheley, S., Anderson, R., Cupples, M. J., Lee Chan, E. C. M. & Morris, V. L., Virolology, in press) have, in fact, demonstrated the presence of separate mRNA species in MHV-infected cells. According to the nomenclature system suggested by Sturman et al. (Journal of Virolology 33, 449–462), we propose that the designations E2, N and E1 be applied to p180, p56 and p24 respectively; furthermore, that PE1 be applied to p22.

REFERENCES


Synthesis of MHV polypeptides


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