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Coronavirus JHM: Coding Assignments of Subgenomic mRNAs

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SUMMARY

Protein synthesis in the murine hepatitis virus JHM-infected cells was temporarily inhibited by hypertonic shock. When the cells were returned to isotonic medium the synthesis of six virus-specific polypeptides, 150K, 65K, 60K, 30K, 23K and 14K was reinitiated simultaneously. Polyadenylated RNA isolated from the cytoplasm or polysomes of infected cells was translated *in vitro* and the products included polypeptides with molecular weights (mol. wt.) of 120000, 60000, 30000, 23000 and 14000. Immunoprecipitation and fingerprinting of [^{35}S]methionine-containing tryptic peptides showed that the 60000 and 23000 mol. wt. products were identical to the 60K and 23K polypeptides found in infected cells; the 120000 mol. wt. product showed identity with the 150K intracellular polypeptide and a virus-specific 120K polypeptide synthesized in tunicamycin-treated cells. Two-dimensional polyacrylamide gel electrophoresis strongly suggested that the 30000 and 14000 mol. wt. products are equivalent to virus-specific 30K and 14K intracellular polypeptides. [^{3}H]Uridine-labelled polyadenylated virus RNA was isolated from infected cells and sedimented in sucrose gradients containing formamide. The distribution in the gradient of each of the previously identified virus RNAs was determined by gel electrophoresis and gradient fractions enriched for each RNA were translated *in vitro*. The 120000, 60000, 30000, 23000 and 14000 mol. wt. polypeptides were found to be encoded by mRNAs 3, 7, 2, 6, and 4 or 5 respectively. These results demonstrate that the virus-specific polypeptides in JHM-infected cells are encoded in separate subgenomic mRNAs and are translated independently. The assignment of coding functions and the known sequence relationships of JHM RNAs permitted a gene order to be deduced.

INTRODUCTION

Coronaviruses infect both animals and humans and are the cause of clinically and economically important diseases. The virions are enveloped, contain a large positive-stranded RNA genome and characteristically three classes of protein: a nucleocapsid protein (50×10^3 to 60×10^3), a matrix protein(s) (20×10^3 to 35×10^3) and a high mol. wt. glycoprotein(s) (90×10^3 to 180×10^3) which constitutes the virion peplomer and is responsible for attachment and cell-to-cell fusion (Siddell *et al.*, 1982; Wege *et al.*, 1982; Collins *et al.*, 1982). In coronavirus-infected cells a 3′ co-terminal 'nested set' of subgenomic RNAs is produced (Stern & Kennedy 1980*a*, *b*; Lai *et al.*, 1981; Spaan *et al.*, 1981, 1982; Cheley *et al.*, 1981; Weiss & Leibowitz, 1981). These RNAs are thought to be mRNAs by several criteria. They are capped (Lai *et al.*, 1982), polyadenylated (Stern & Kennedy, 1980*a*; Lai *et al.*, 1981; Wege *et al.*, 1981; Cheley *et al.*, 1981; Spaan *et al.*, 1981) and are found in association with polysomes (Spaan *et al.*, 1981; Wege *et al.*, 1981; Lai *et al.*, 1982). Some of these RNAs have also been translated *in vitro* to yield products identified as virus-specific polypeptides (Siddell *et al.*, 1980; Rottier *et al.*, 1981*a*; Cheley *et al.*, 1981; Stern *et al.*, 1982; Leibowitz & Weiss, 1981).

The MHV-JHM strain of murine hepatitis virus is of particular interest in that it has the ability to induce central nervous system disorders in rodents and has been used as a model for virus-induced demyelination (Nagashima *et al.*, 1978, 1979). The virion has a genomic RNA of

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approximate mol. wt. 6×10^6 (Leibowitz *et al.*, 1981; Wege *et al.*, 1981) and six major proteins which conform to the characteristic coronavirus pattern (Siddell *et al.*, 1981*a*; Lai & Stohlman, 1981). In infected cells seven virus-specific RNA species, which are related in the predicted 'nested set' fashion (Leibowitz *et al.*, 1981; Wege *et al.*, 1981; Cheley *et al.*, 1981), have been detected and six intracellular polypeptides, whose relationship to the virion proteins has been established by tryptic peptide fingerprinting (Siddell *et al.*, 1981*a*, Siddell, 1982), have also been characterized.

Earlier studies (Siddell *et al.*, 1980) demonstrated that the smallest MHV-JHM subgenomic RNA (RNA 7) encodes the virion nucleocapsid protein, and this result has been confirmed for the related MHV-A59 by Rottier *et al.* (1981*a*), Cheley *et al.* (1981) and Leibowitz & Weiss (1981). The same data also suggested that at least two more of the subgenomic RNAs, namely RNA 6 (Siddell *et al.*, 1980; Rottier *et al.*, 1981*a*) and RNA 3 (Rottier *et al.*, 1981*a*) function as mRNAs, although their translation products were only tentatively identified as virus-specific by electrophoretic and immunological procedures.

In this report, we have extended these studies by demonstrating the mRNA function of five of the six MHV-JHM subgenomic RNAs and identifying their translation products by one- or two-dimensional gel electrophoresis, immunoprecipitation and tryptic peptide fingerprinting. In addition, we have demonstrated that these mRNAs are independently translated *in vivo*. As the sequence relationship of MHV-JHM RNAs is known (Leibowitz *et al.*, 1981), these results allow an almost complete gene order to be deduced for MHV-JHM.

METHODS

Virus and cells. Sac(-) cells were grown as suspension or monolayer cultures and MHV-JHM stocks were obtained and propagated as described previously (Siddell *et al.*, 1980).

Intracellular labelling of polypeptides

After salt treatment. Four Petri dishes (1, 2, 3 and 4) of Sac(-) cells were infected at an m.o.i. of 5 with MHV-JHM as described previously (Siddell *et al.*, 1980) and incubated for 9 h in minimal essential medium (MEM) containing 5% foetal calf serum (FCS). The infected cells were then moved either to MEM without methionine but containing 2% dialysed FCS (dishes 1 and 3) or to the same medium containing an additional 225 mM-NaCl (dishes 2 and 4) and incubated for 20 min at 37 °C. The cells were then moved to either MEM without methionine containing 2% dialysed FCS and 100 μCi/ml of [^{35}S]methionine (SJ204; Amersham) (dishes 1 and 2), or to the same medium containing an additional 225 mM-NaCl (dishes 3 and 4) and incubated for 30 s at 37 °C. The labelling medium was then removed from all dishes and MEM containing 5% FCS was added and the cells were incubated at 37 °C for 10 min. Cytoplasmic lysates were then prepared at 4 °C as previously described (Siddell *et al.*, 1981*a*).

After tunicamycin treatment. Two Petri dishes of Sac(-) cells were infected at an m.o.i. of 5 with MHV-JHM as previously described (Siddell *et al.*, 1980) and incubated for 9 h in MEM containing 5% FCS. The infected cells were then moved to MEM containing 5% FCS, or MEM containing 5% FCS and 1 μg/ml tunicamycin (Eli Lilly, Indianapolis, Ind., U.S.A.) and incubated at 37 °C for 15 min. Next, the cells were incubated either in MEM without methionine containing 2% dialysed FCS or in the same medium containing 1 μg/ml tunicamycin for 15 min at 37 °C. Finally, the cells were moved to MEM without methionine containing 2% dialysed FCS and 100 μCi/ml [^{35}S]methionine or the same medium containing 1 μg/ml tunicamycin and incubated for 30 min at 37 °C. Cytoplasmic lysates were then prepared at 4 °C as described previously (Siddell *et al.*, 1981*a*).

Immunoprecipitation, gel electrophoresis and tryptic peptide mapping. Immunoprecipitation of cytoplasmic lysates with hyperimmune serum raised against purified virus was performed as previously described (Siddell *et al.*, 1981*a*). For one-dimensional electrophoresis, cytoplasmic lysates, immunoprecipitates or the products of *in vitro* translations were mixed with electrophoresis sample buffer (Siddell *et al.*, 1980), boiled for 2 min and electrophoresed on 15% discontinuous SDS–polyacrylamide gels as described by Laemmli (1970). For two-dimensional electrophoresis, the products of *in vitro* translations were treated with pancreatic ribonuclease (Siddell *et al.*, 1981*a*) and subjected to non-equilibrium pH gradient electrophoresis (NEPHGE) or isoelectric focussing (IEF) in the first dimension and SDS–polyacrylamide gradient gel electrophoresis (PAGE) in the second dimension as described by O'Farrell (1975) and O'Farrell *et al.* (1977) with the modifications described in Siddell *et al.* (1981*a*). All gels were stained, dried and exposed for autoradiography or fluorography (Laskey & Mills, 1975).

The procedures used to produce [^{35}S]methionine-containing tryptic peptide fingerprints of virus-specific proteins synthesized in infected cells or in cell-free protein synthesis have been described in detail (Siddell *et al.*, 1980; Siddell, 1982). All tryptic peptide fingerprints presented here were produced from polypeptides which had been immunoprecipitated with anti-JHM serum before electrophoresis.

Intracellular labelling of virus specific RNA. Suspension cultures of Sac(-) cells (4×10^7) were infected with MHV-JHM at an m.o.i. of 5 as previously described (Siddell *et al.*, 1980) and incubated for 4 h in Joklik's modification of MEM containing 5% FCS (spinner medium) at 37 °C. The cells were then moved into spinner medium containing 1 μg/ml actinomycin D (Sigma) and incubated at 37 °C. After 45 min, 1 mCi of [^{3}H]uridine (TRK 178; Amersham) was added to the medium and 2 h later, polyadenylated cytoplasmic RNA was isolated.

Isolation of polyadenylated RNA. Unlabelled polyadenylated cytoplasmic RNA was isolated from 5×10^8 Sac(-) cells which had been infected with MHV-JHM at 0·3 $TCID_{50}$/cell, or mock-infected, 16 h previously (Siddell *et al.*, 1980) and incubated in spinner medium at 37 °C. Briefly, the cells were pelleted and washed twice in Tris-buffered saline pH 7·4. The cell pellet was resuspended in 7 vol. of ice-cold 20 mM-Tris–HCl pH 7·5, 100 mM-NaCl, 5 mM-$MgCl_2$ and kept for 5 min at 4 °C. Nonidet P40 was added to a concentration of 0·1% and the lysate was immediately centrifuged at 1500 *g* for 5 min at 4 °C. Polyvinyl sulphate and SDS were added to the cytoplasmic lysate, to 100 μg/ml and 0·5% respectively, and the RNA was extracted by repeated shaking with phenol–chloroform–isoamyl alcohol (50 : 50 : 1) and precipitated with alcohol. Polyadenylate-containing RNA was selected from this material by two cycles of chromatography on polyuridylic acid–Sepharose (Lindberg & Persson, 1974).

Unlabelled polyadenylated RNA from the polysomes of Sac(-) cells infected as described above was prepared by a procedure described in detail elsewhere (Siddell *et al.*, 1980). Essentially, polysomes from an infected-cell lysate were isolated by sucrose gradient centrifugation followed by pelleting through a sucrose cushion. RNA released from the polysomes by puromycin treatment was recovered after separation from polysomal subunits on sucrose gradients, deproteinized by repeated shaking with phenol–chloroform–isoamyl alcohol and recovered by alcohol precipitation. The RNA was then subjected to two cycles of chromatography on polyuridylic acid–Sepharose.

In vitro *translation.* Rabbit reticulocyte lysates were prepared and used as described by Pelham & Jackson (1976) except that amino acids (minus methionine) were added to 0·2 mM each, and 2-aminopurine was added to a concentration of 6 mM. A messenger-dependent L-cell lysate was prepared and used as described by Paucha *et al.* (1978) and Dahl & Dickson (1979).

Fractionation of RNA. Cytoplasmic polyadenylated RNA was fractionated on 5 to 20% sucrose gradients containing 50% formamide as described by Smith *et al.* (1976). Samples were denatured in 60% formamide, 6 mM-Tris–HCl pH 7·6, 0·6 mM-EDTA at 37 °C for 10 min before centrifugation. Analysis of RNA was performed by electrophoresis in 1% agarose gels after denaturation with glyoxal–dimethyl sulphoxide as described by McMaster & Carmichael (1977). Gels were dried and subjected to fluorography at −80 °C (Laskey & Mills, 1975). ^{3}H-labelled ribosomal RNA was prepared from uninfected L-cells.

RESULTS

When coronavirus JHM-infected Sac(-) cells are exposed to medium containing 345 mM-NaCl at 37°C, protein synthesis is reduced by over 95% within 20 min (Fig. 1, lanes 1 and 4). When the cells are returned to isotonic medium, a complete and synchronous recovery of the initiation of protein synthesis can be achieved (Saborio *et al.*, 1974). Assuming that the rate of translation is about 28000 mol. wt. protein/min (Clegg, 1975), the synchronously reinitiated proteins of greater than about 14000 mol. wt. can be identified upon return to isotonic medium by pulse-labelling the cells for 30 sec followed by a 10 min chase period to allow completion of larger (up to about 280000 mol. wt.) proteins. Fig. 1 (lanes 1 and 2) shows that in JHM-infected cells polypeptides of 14000, 23000, 30000, 60000, 65000 and 150000 mol. wt. can be identified as independently initiated. The mol. wt. of these polypeptides corresponds exactly to those of the six previously identified virus-specific intracellular polypeptides (Siddell *et al.*, 1981*a*).

Further identification of four polypeptides (23000, 60000, 65000, 150000 mol. wt.) as intracellular virus polypeptides is provided by their specific immunoprecipitation with antiserum raised against purified virus (Fig. 1, lanes 2a and 2b). These results are in agreement with conclusions reached earlier by Cheley & Anderson (1981), but extend their observations to include three further MHV-specific intracellular polypeptides and to identify the simultaneously reinitated virus structural proteins by immunological as well as electrophoretic procedures.

If the synthesis of each of the JHM intracellular polypeptides is independently initiated, it is most likely that multiple mRNA species exist. Therefore, polyadenylated RNAs were isolated from infected cell cytoplasm and polysomes and translated in cell-free systems made from L-cell or rabbit reticulocyte lysates. Fig. 2(*a*, *b*) shows that in both systems 60000 and 23000 mol. wt. polypeptides were the major products of translation directed by either cytoplasmic or polysomal polyadenylated RNA. In the reticulocyte system, a third major product of 120000 mol. wt. was observed. (Fig. 2*b*, lanes 3 and 4). All three of these products of translation *in vitro* were

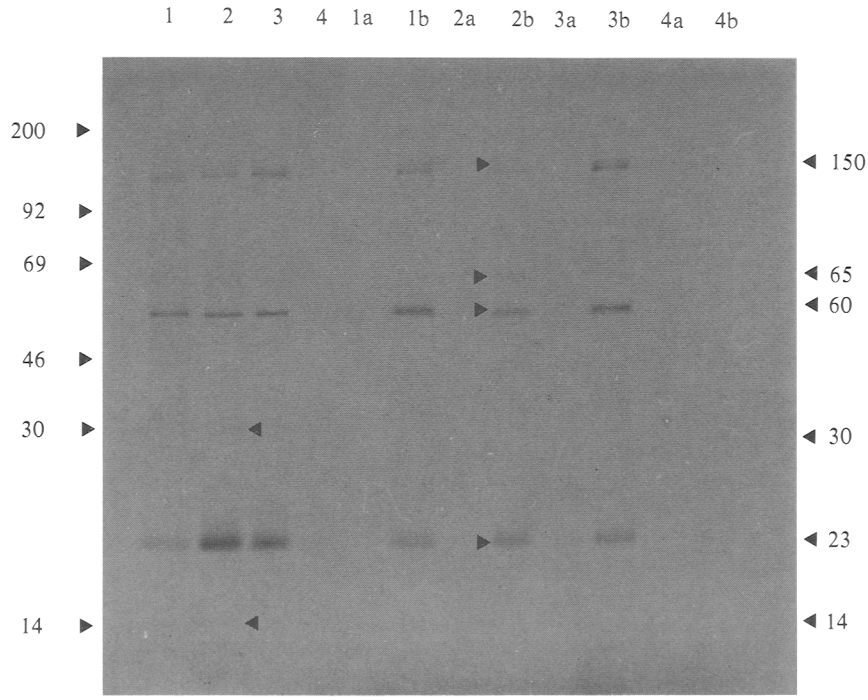


Fig. 1. Synchronous reinitiation of protein synthesis in MHV-JHM-infected cells. Infected cells were blocked with hypertonic salt and then returned to isotonic conditions while the intracellular polypeptides were labelled as described in Methods (lane 2). As controls, infected cells were pulse-labelled under isotonic conditions without having been previously exposed to hypertonic salt (lane 1) or cells were labelled under hypertonic salt conditions with (lane 4) or without (lane 3) having been previously exposed to hypertonic salt. Immunoprecipitation with control or anti-JHM serum respectively of the samples shown in lanes 1 to 4 are shown in lanes 1a, 1b, 2a, 2b, 3a, 3b and 4a, 4b. The gel was fluorographed for 180 days. Closed triangles indicate the positions of virus-specific polypeptides, whose mol. wt. is shown at the right. The positions of mol. wt. standards are shown at the left.

specifically immunoprecipitated with antiserum raised against purified virions (Fig. 2*a*, lanes 3a and 3b; 2*b*, lanes 4a and 4b) and their identity as virion structural proteins was further confirmed by tryptic peptide fingerprinting.

Fig. 3 shows the tryptic peptide fingerprints of the 60000 and 23000 mol. wt. products; they are identical to the previously described fingerprints of the virion proteins pp60 and p23, and their intracellular counterparts, 60K and 23K (Siddell, 1982). The tryptic peptide fingerprint of the 120000 mol. wt. product shares identity with that of the 150K intracellular polypeptide, a precursor to the virion proteins gp170 and gp98 (Siddell, 1982).

As the MHV 150K intracellular polypeptide is glycosylated (Rottier *et al.*, 1981*b*; S. G. Siddell, unpublished), it seemed likely that the difference between the size of the 120000 mol. wt. product synthesized *in vitro* and the 150K polypeptide synthesized *in vivo* was due to this modification. Fig. 4 shows that in Sac(-) cells infected with MHV-JHM and treated with tunicamycin, the synthesis of the intracellular 150K species was no longer detected and instead a new polypeptide of 120000 mol. wt. was synthesized (Cheley & Anderson, 1981; Niemann & Klenk, 1981). This polypeptide was specifically immunoprecipitated by anti-JHM serum (Fig. 4, lanes 1a and 1b) and fingerprinting of its [^{35}S]methionine-containing tryptic peptides confirmed its identity as the apoprotein to the 150K species (Fig. 5).

In addition to the major products made by JHM mRNA *in vitro*, it was also possible to detect a 30000 mol. wt. product (Fig. 2*a*, lane 2) and a 14000 mol. wt. polypeptide (the latter is not readily seen in Fig. 2, but can be clearly seen in the translation products of size-fractionated mRNA in Fig. 8). Neither of these polypeptides was immunoprecipitated by anti-JHM serum

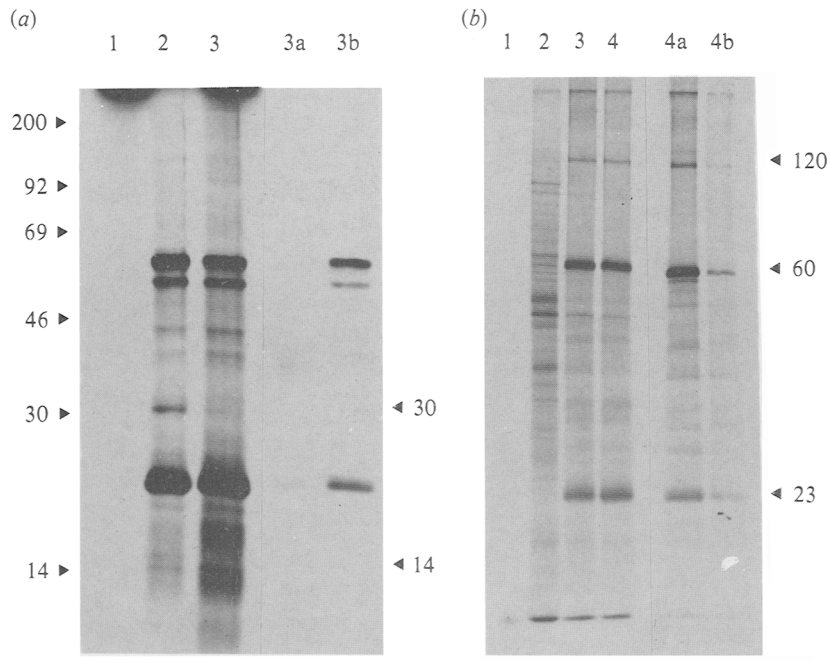


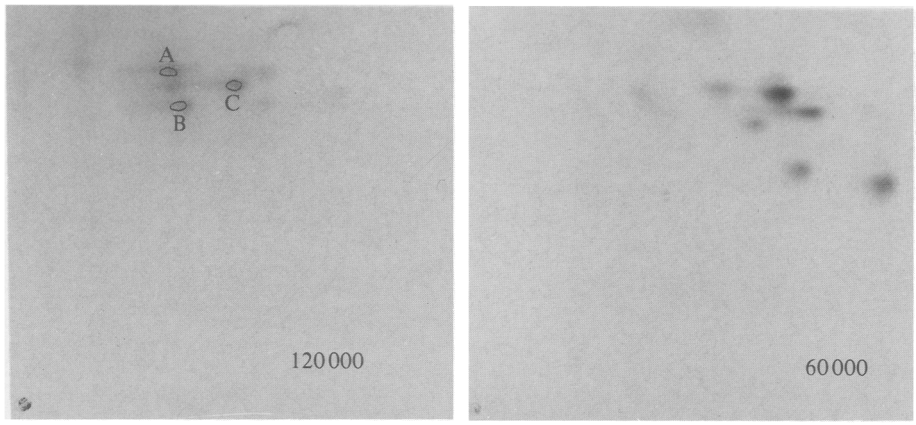
Fig. 2. *In vitro* translation of polyadenylate-containing cytoplasmic and polysomal RNA from MHV-JHM infected cells. (*a*) Translation in L-cell lysate: lane 1, no added RNA; lane 2, cytoplasmic RNA; lane 3, polysomal RNA; immunoprecipitation of the sample shown in lane 3 with control (lane 3a) or anti-JHM (lane 3b) serum. (*b*) Translation in reticulocyte lysate: lane 1, no added RNA; lane 2, uninfected cell cytoplasmic polyadenylated RNA; lane 3, cytoplasmic RNA; lane 4, polysomal RNA; immunoprecipitation of the sample shown in lane 4 with control (lane 4b) or anti-JHM (lane 4a) serum.

(Fig. 2) but their designation as virus-specific was strongly supported by their electrophoretic behaviour on two-dimensional PAGE.

Fig. 6 shows both IEF and NEPHGE two-dimensional PAGE of the products directed by cytoplasmic polyadenylated RNA from infected cells as translated in the L-cell system. The electrophoretic behaviour of the minor 30000 and 14000 mol. wt. species in both dimensions is characteristic of the previously described intracellular 30K and 14K polypeptides (Siddell *et al.*, 1981 *a*), and thus confirms their identity. The major products *in vitro* of 60000 and 23000 mol. wt. also display the electrophoretic behaviour of their intracellular counterparts (Siddell *et al.*, 1981 *a*). However, it is clear that the nucleocapsid polypeptide synthesized *in vitro* (60000 mol. wt.) is heterogeneously charged, a property we did not detect with the intracellular 60K protein (Siddell *et al.*, 1981 *a*, *b*). Very little of the 120000 mol. wt. product was made *in vitro* in the L-cell system and it was not detected by two-dimensional PAGE.

Finally, Fig. 2 also shows that the 30000 mol. wt. product is made *in vitro* in greatly reduced amounts when polysomal polyadenylated RNA is translated (Fig. 2*a*, lanes 2 and 3). This observation correlates with our earlier finding that the synthesis of the 30K species is difficult to detect *in vivo* (Siddell *et al.*, 1981 *a*) and suggests that a translational control is exerted over the synthesis of this polypeptide in the cell.

In order to demonstrate the independent nature of each virus-specific mRNA, [^{3}H]uridine-labelled polyadenylated RNA was isolated from the cytoplasm of actinomycin D-treated infected cells and sedimented in sucrose gradients containing formamide (Fig. 7*a*). The gradients were fractionated and samples from each fraction were analysed by electrophoresis. The different-sized virus RNA species (RNAs 1 to 7) sedimented to different positions in the gradient, with the larger RNAs sedimenting farther. The sedimentation values for the major virus RNAs were determined in these gradients as: 1, 45S to 50S; 2, 30S; 3, 27S; 4, and 5, 21S to 22S; 6, 19S and 7, 17S (Fig. 7*b*). It was thus possible to identify the virus RNA species in these



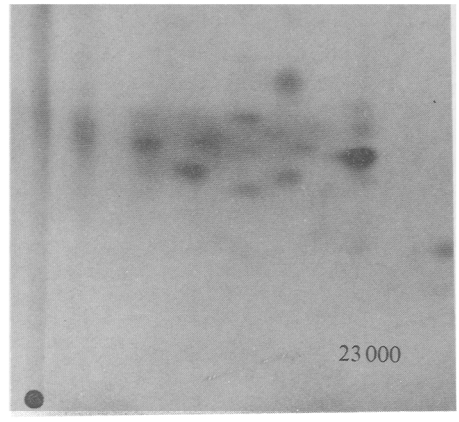


Fig. 3

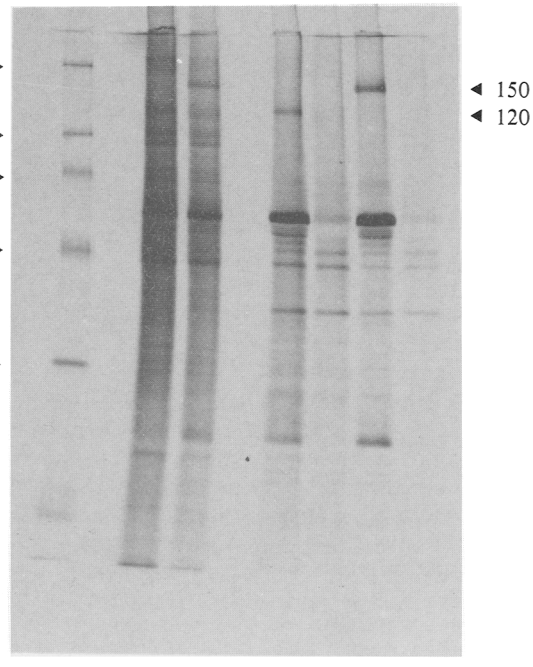


Fig. 4

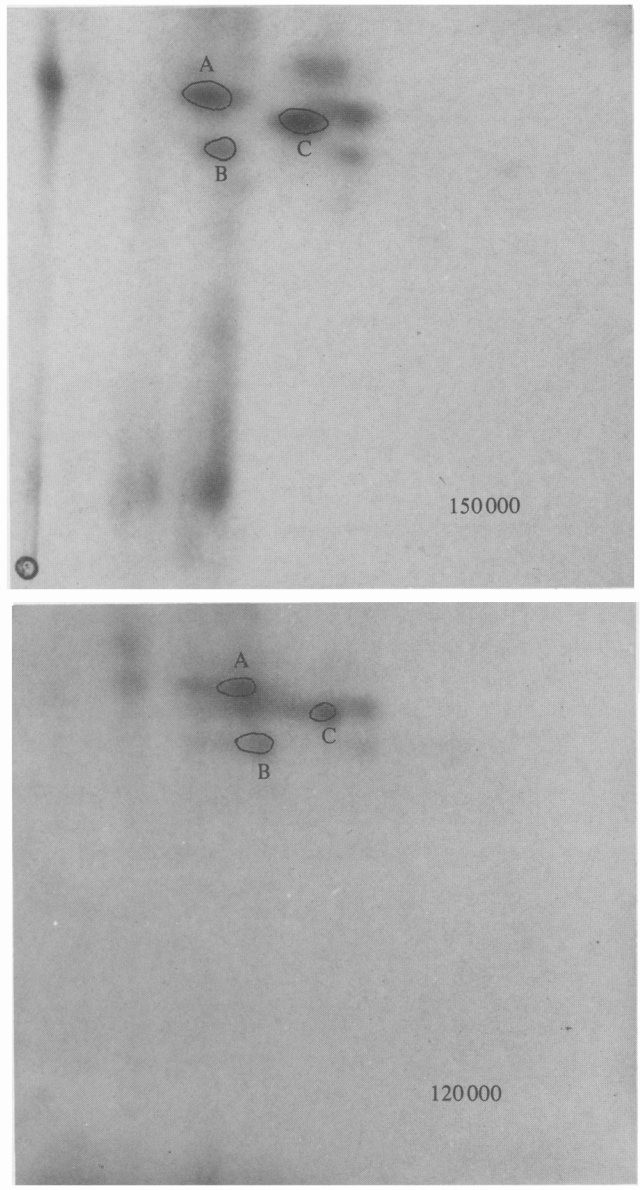


Fig. 5. Tryptic peptide fingerprinting of the 120000 mol. wt. MHV-JHM polypeptide synthesized in tunicamycin-treated cells. The [^{35}S]-methionine-containing tryptic peptide fingerprint of the 120000 mol. wt. virus-specific polypeptide synthesized in tunicamycin-treated cells, or the 150K polypeptide synthesized in untreated cells were prepared from immunoprecipitated samples as described in Methods.

Fig. 3. Tryptic peptide fingerprinting of MHV-JHM polypeptides synthesized *in vitro*. [^{35}S]Methionine-containing tryptic peptide fingerprints of the 120000, 60000 and 23000 mol. wt. products synthesized in a reticulocyte lysate and immunoprecipitated with anti-JHM serum were prepared as described in Methods.

Fig. 4. MHV-JHM-specific polypeptides synthesized in tunicamycin-treated cells. Cells were infected and pulse-labelled with (lane 1) or without (lane 2) treatment with tunicamycin as described in Methods. Immunoprecipitates of the samples shown in lanes 1 and 2 with anti-JHM or control serum respectively are shown in lanes 1a, 1b, 2a and 2b.

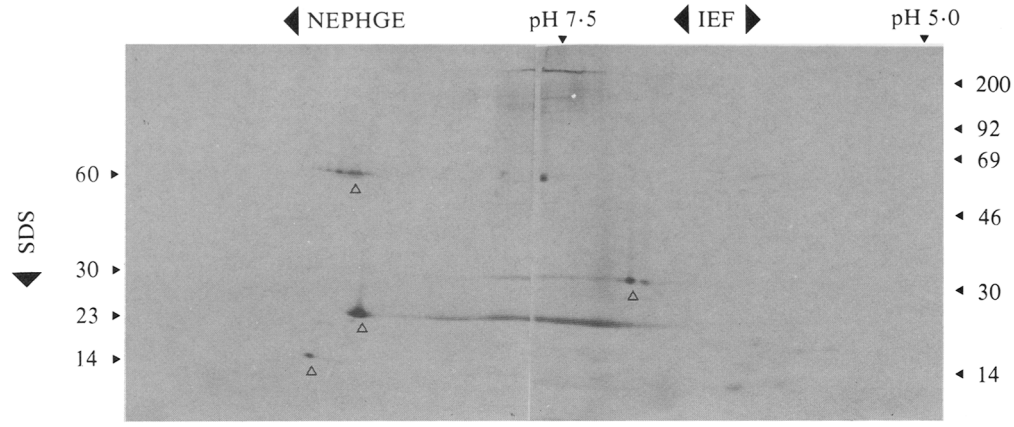
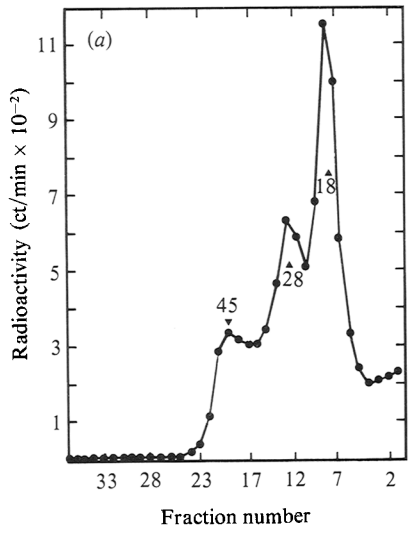
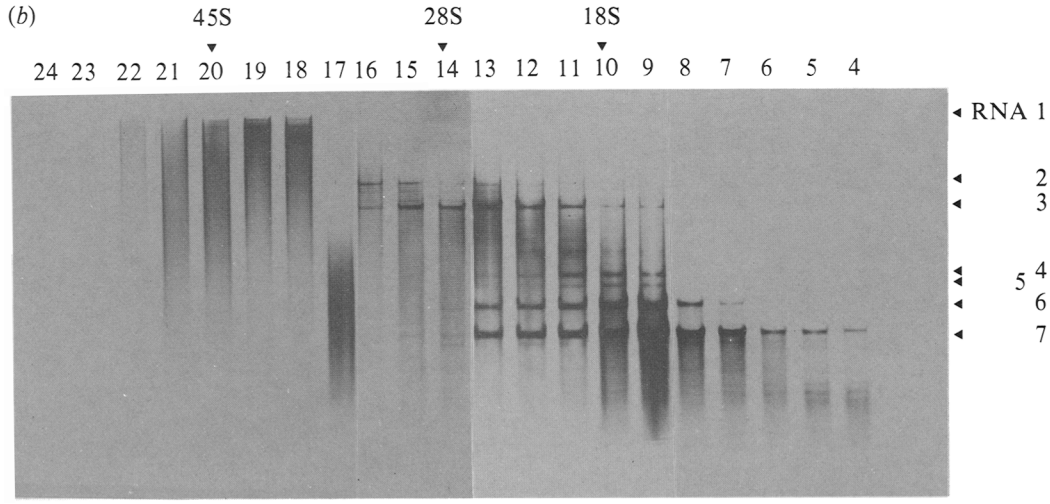


Fig. 6. Two-dimensional gel electrophoresis of polypeptides synthesized in a L-cell lysate directed by cytoplasmic polyadenylated RNA from MHV-JHM-infected cells. Open triangles indicate the positions of virus-specific polypeptides.





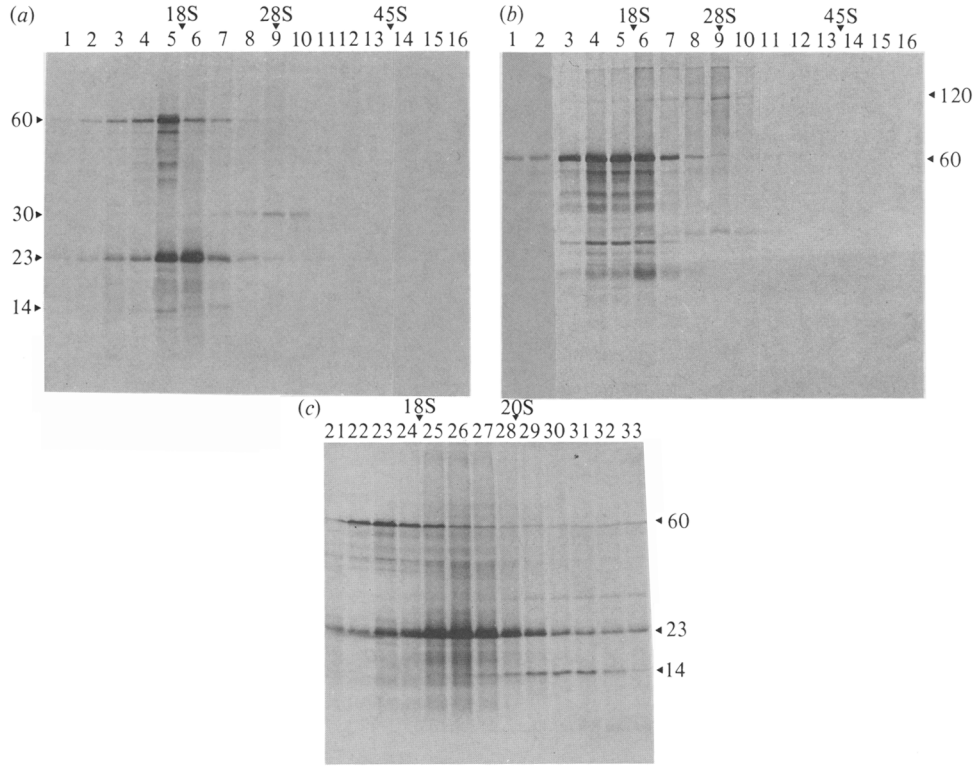


Fig. 8. *In vitro* translation of size-fractionated cytoplasmic polyadenylated RNA from MHV-JHM-infected cells. Cytoplasmic polyadenylated RNA was isolated from MHV-JHM-infected cells. 20 μg of RNA were sedimented in 12 ml gradients of 5 to 20% sucrose containing 50% formamide for 30 h at 20000 *g* at 20 °C in a SW41 rotor. The gradients were separated into 16 (*a*, *b*) or 80 fractions (*c*). The RNA was recovered from each fraction and aliquots were translated in a L-cell lysate (*a*, *c*) or translated in a reticulocyte lysate (*b*) and immunoprecipitated with anti-JHM serum before electrophoresis.

gradients. Unlabelled, infected-cell cytoplasmic polyadenylated RNA was fractioned on similar gradients, recovered and translated *in vitro* and the virus RNA which encoded each virus polypeptide could be determined. Fig. 8(*a*) shows that the RNAs encoding the 60000, 23000 and 30000 mol. wt. products sediment at 17S, 19S and 30s respectively, and can thus be identified as RNA 7, RNA 6 and RNA 2. The mRNA encoding the 120000 mol. wt. polypeptide could be identified as sedimenting at 27S, i.e. RNA 3, by translating the same fractions of RNA in the reticulocyte system and immunoprecipitating the products synthesized *in vitro* (Fig. 8*b*). It could be shown, by collecting a greater number of fractions from the gradient, that the mRNA encoding the 14000 mol. wt. product sedimented at 21S to 22S (Fig. 8*c*). Because two major virus RNA species sediment at this value (RNA 4 and RNA 5), it is not possible to conclude at the moment which of these RNAs encodes this protein. Nevertheless these results demonstrate that,

Fig. 7. Gradient fractionation of MHV-JHM intracellular polyadenylated RNA. ^{3}H-Labelled cytoplasmic polyadenylated RNA was isolated from actinomycin D-treated MHV-JHM infected cells. 200000 ct/min of RNA were sedimented in a 12 ml gradient of 5 to 20% sucrose containing 50% formamide for 20 h at 20000 *g* (av.) at 20 °C in a SW41 rotor. The gradient was separated into 37 fractions, and (*a*) aliquots were counted directly; the remainder of each fraction (*b*) was precipitated with 5 μg of carrier tRNA by ethanol, treated with DMSO–glyoxal and electrophoresed on 1% agarose gels as described in Methods. The positions of ribosomal RNA markers (18S, 28S and 45S) were determined in a parallel gradient.

with this exception, it is possible to correlate each major subgenomic MHV-JHM mRNA with its translation product.

Although fractions containing cytoplasmic polyadenylated RNA of genome size have been recovered and translated, no product directed *in vitro* by mRNA of this size could be detected. A number of possible explanations for this result can be proposed. For example, the reticulocyte cell-free system used in this study was capable of synthesizing polypeptides of 200000 mol. wt., but possibly larger polypeptides could not be made. Alternatively, efficient *in vitro* translation of this RNA may require conditions different from those used in these experiments. These possibilities are being investigated. A further unexpected result was the inability to detect the synthesis of a polypeptide *in vitro* which correlated with the intracellular 65K virus-specific polypeptide. This polypeptide is found in highly purified virion preparations (Siddell *et al.*, 1982) and tryptic peptide mapping shows that it is unrelated to the other virus polypeptides. The intracellular polypeptide, 65K, is reinitiated simultaneously with the other virus-specific polypeptides after a salt block and is therefore presumably a primary translation product. Why it has not been possible to detect the synthesis of a corresponding polypeptide *in vitro* is at the moment unexplained.

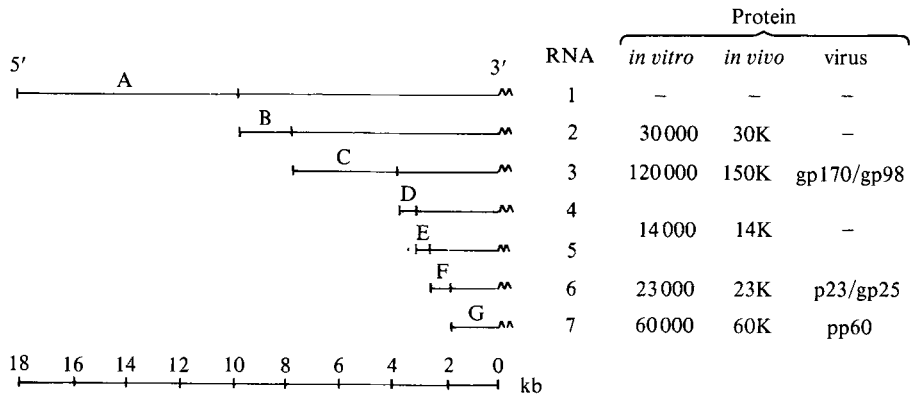
DISCUSSION

The results presented here demonstrate the functional independence of MHV-JHM mRNAs *in vitro* and *in vivo*. Furthermore, with one ambiguity, they identify the translation product of each of the major subgenomic mRNAs found in infected cells as one of the virus-specific intracellular proteins. The results therefore strongly support the model for coronavirus replication which was emerged from the studies of many laboratories (Stern & Kennedy, 1980*a*, *b*; Stern *et al.*, 1982; Lai *et al.*, 1981, 1982; Spaan *et al.*, 1981, 1982; Rottier *et al.*, 1981*a*, *b*; Cheley *et al.*, 1981; Cheley & Anderson, 1981; Leibowitz *et al.*, 1981; Wege *et al.*, 1981; Siddell *et al.*, 1980; 1981; Siddell, 1982; for review, see Siddell *et al.*, 1982). According to this model, the virus genetic information is expressed through multiple RNAs of genomic and subgenomic size, which form a 3′ co-terminal 'nested set'. Each RNA functions as a mRNA and encodes one protein. Comparison of the size of each mRNA with its translation product suggests that the information expressed lies within those 5′ sequences of each RNA which are not found in the next-smallest RNA. Presumably the redundant coding information in each RNA (excluding the smallest) remains untranslated because internal initiation sites are not used (Kozak, 1981).

A verification of this model will clearly require sequence analysis of coronavirus nucleic acids and proteins. However, the supporting evidence is now considerable and it seems reasonable to predict a gene order for MHV-JHM on this basis. Fig. 9 shows the arrangement of MHV-JHM subgenomic mRNA as it has been elucidated by T_1-oligonucleotide mapping and the use of cDNA probes (Leibowitz *et al.*, 1981; Cheley *et al.*, 1981). The *in vitro* translation product encoded by each mRNA is shown, its equivalent in the infected cell and its virion counterpart (see above; Siddell, 1982). Thus, the gene order for MHV-JHM can be deduced as 5′–NSA–NSB(30K)–gp170/gp98–NSD/NSE(14K)–p23/gp25–pp60–3′. [The nomenclature used here follows that given in Siddell *et al.* (1982), where NSA, NSB, etc represent non-structural polypeptides A, B, etc.]

A number of comments on the MHV-JHM replication strategy can be made. Firstly, the genes encoding the coronavirus structural proteins appear to be clustered towards the 3′ half of the genomic information. Secondly, the replication strategy appears to be a particularly flexible one, allowing for the control of the synthesis of any individual protein at the levels of both transcription and translation. However, the strategy does not appear to be particularly economical, as a great deal of redundant information is contained within the subgenomic mRNA species. Thirdly, as with positive-stranded viruses such as alphaviruses (Kääriänen & Söderlund, 1978) and certain plant viruses (Fraenkel-Conrat *et al.*, 1977), the MHV-JHM subgenomic mRNAs extend inwards from the 3′ end of the genome. Whether this reflects evolutionary relationships or constraints imposed by the replicative mechanism of the viral mRNAs is not clear.

The major question which has not been answered in this paper is the coding function of the MHV-JHM genome-sized RNA. As neither the host cell nucleus nor continued host cell



RNA	*in vitro*	*in vivo*	virus
1	–	–	–
2	30000	30K	–
3	120000	150K	gp170/gp98
4			
5	14000	14K	–
6	23000	23K	p23/gp25
7	60000	60K	pp60

Fig. 9. Coding assignments of MHV-JHM subgenomic mRNAs.

transcription are reported to be necessary for MHV replication (Brayton *et al.*, 1981; Wilhelmsen *et al.*, 1981), the incoming MHV genome would presumably encode a RNA polymerase, necessary to synthesize negative-stranded template required for the subsequent production of the subgenomic mRNAs. To date there have been only three reports of virus-specific RNA-dependent RNA polymerase activity isolated from coronavirus-infected cells (Dennis & Brian, 1982; Mahy *et al.*, 1982; Brayton *et al.*, 1982). The polypeptide components of the enzyme have not, however, been identified and it is not known whether the gene encodes a single polypeptide, or a polypeptide which is cleaved to yield smaller polypeptides. Also the synthesis of any virus-specific proteins at the earliest stages of infection, i.e. prior to the onset of mRNA production, have not been detected. Leibowitz & Weiss (1981) have reported the translation *in vitro* of genome-sized RNA from MHV virions to yield high molecular weight polypeptides (over 200000 mol. wt.) but it is not yet possible to identify these polypeptides as polymerase components or their precursors. The observations that genome-sized RNA is found associated with polysomes at late stages of infection (Spaan *et al.*, 1981; Wege *et al.*, 1981; Lai *et al.*, 1982) and that even at the later stages of infection virus RNA synthesis is reduced when *de novo* protein synthesis is inhibited by anisomycin (Mahy *et al.*, 1982) raises the possibility that different or modified polymerase activities are responsible for negative and positive strand virus RNA synthesis. Whether differences in the genomic RNA and polysomal RNA 1, differences in the synthesis and processing of the gene A product, or modification of the polymerase activity by virus or host cell products dictates the polarity of RNA strand synthesis remains to be elucidated.

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