

## Coronavirus JHM: Cell-Free Synthesis of Structural Protein p60

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Sac(-) cells infected with murine coronavirus strain JHM shut off host cell protein synthesis and synthesized polypeptides with molecular weights of 150,000, 60,000, and 23,000. The 60,000- and 23,000-molecular-weight polypeptides comigrated with virion structural proteins p60 and p23, and the 60,000-molecular-weight protein was identified as p60 by tryptic peptide fingerprinting. Polyadenylate-containing RNA [poly(A) RNA] extracted from the cytoplasm of infected cells directed the synthesis of both 60,000- and 23,000-molecular-weight polypeptides in messenger-dependent cell-free systems derived from mouse L-cells and rabbit reticulocytes. The reticulocyte system also synthesized a 120,000-molecular-weight polypeptide that was specifically immunoprecipitated by antiserum raised against JHM virions. The identity of the 60,000- and 23,000-molecular-weight in vitro products was established by comigration with virion proteins, immunoprecipitation, and in the case of p60, tryptic peptide fingerprinting. The cytoplasmic poly(A) RNAs which encoded p60 and p23 sedimented in sucrose-formamide gradients at 17S and 19S, respectively, and were clearly separable. These RNAs were among the major poly(A) RNA species synthesized in the cytoplasm of actinomycin D-treated cells late in infection, and the in vitro translation of size-fractionated RNA released from polysomes confirmed that they represent physiological mRNA's. These results suggest that the expression of the coronavirus JHM genome involves more than one subgenomic mRNA.

Coronaviruses cause a variety of diseases in both animals and humans. They are associated with respiratory and enteric diseases in humans, bronchitis in birds, transmissible gastroenteritis and encephalitis in pigs, and demyelinating encephalitis and hepatitis in rodents (11, 20; J. A. Robb and C. W. Bond, *in* H. Fraenkel-Conrat and R. R. Wagner, ed., *Comprehensive Virology*, vol. 14, in press).

The neurotropic murine coronavirus JHM (JHMV) induces a variety of central nervous system disorders in mice and rats and can be used as a model for virus-induced demyelination (4, 8, 12, 13). JHM virions are pleomorphic spherical particles about 100 nm in diameter with characteristic club-shaped surface projections (11). The virus matures, as do other coronaviruses, by budding from internal cellular membranes and not from the plasma membranes (11). JHM virions have been characterized recently. The genome is an infectious single-stranded RNA with a molecular weight of about  $5.4 \times 10^6$  (7, 21). The RNA is a continuous strand, and at least one-third of the molecules contain polyadenylate [poly(A)] sequences (7, 21). The virion contains six major proteins, four of which are glycosylated (2, 22). Subviral particles produced by treatment of the virus with

Nonidet P-40 include RNA and one of the major structural proteins of the virus, p60.

In contrast, there is very little information on the replication of JHMV (2, 16; Robb and Bond, in press). We have, therefore, initiated studies on the synthesis of viral RNAs and proteins in infected tissue culture cells. In this paper we report on experiments which demonstrate the in vitro translation and size of the mRNA which encodes the major nucleocapsid protein of the virus, p60. The same experiments also provide information on the synthesis of another virion protein, p23, and indicate the involvement of more than one subgenomic mRNA in the replication strategy of coronaviruses.

### MATERIALS AND METHODS

**Cells and virus.** Sac(-) cells are a permanent murine Moloney sarcoma cell line (23). The cells are grown at 37°C either in suspension with the Joklik modification of minimal essential medium containing 5% fetal bovine serum (Spinner medium) or as a monolayer with minimal essential medium containing 5% fetal bovine serum (medium). We obtained JHMV from L. P. Weiner, Johns Hopkins University, Baltimore, Md., in an homogenate of suckling mouse brain. The virus was propagated twice in suckling mouse brain, isolated, plaque purified four times on L929 cells, and then grown in Sac(-) cells at low multiplic-

ities of infection (MOI). Virus stocks were prepared by infecting monolayers of Sac(-) cells at an MOI of 0.2 50% tissue culture infective dose (TCID<sub>50</sub>) per cell and harvesting the medium 20 h later when 75 to 100% of the cells had formed syncytia. The titer of virus in the clarified medium was between 10<sup>7</sup> and 10<sup>8</sup> TCID<sub>50</sub>/ml.

**Radioactive labeling and purification of virus.** Virus diluted with Puck saline was absorbed in suspension for 20 min at 37°C to 5 × 10<sup>8</sup> Sac(-) cells at an MOI of 0.2 TCID<sub>50</sub>/cell. Unabsorbed virus was removed by centrifuging the cells and suspending them in Spinner medium. This medium was harvested 20 h later and clarified by centrifugation, and the virus was purified as described by Wege et al. (22). Purified virus for gel electrophoresis was stored at -20°C in 50 mM Tris-hydrochloride, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol.

Virus labeled with [<sup>35</sup>S]methionine was prepared by combining the purification of unlabeled virus as described above with clarified medium taken from monolayer cultures infected 12 h previously with JHMV at an MOI of 6 TCID<sub>50</sub>/cell. At between 7 and 10 h after infection, the medium on the monolayers was replaced by medium containing 1/20 the usual amount of methionine, 2% dialyzed fetal bovine serum, and 200 μCi of [<sup>35</sup>S]methionine (SJ204, Amersham Buchler, Braunschweig, West Germany) per ml.

**Labeling of infected-cell proteins. (i) Pulse-labeling.** JHMV, or medium in the case of mock-infected cells, was absorbed to monolayers of Sac(-) cells grown in 5-cm petri dishes for 45 min at 37°C at an MOI of 6 TCID<sub>50</sub>/cell. Unabsorbed virus was removed by washing with medium, and the infected or mock-infected cells were maintained in medium at 37°C. At the times indicated in Fig. 1, the medium was replaced by medium with 1/10 the usual concentration of essential amino acids and without serum (1/10 amino acid medium). After 15 min, this medium was replaced by 1/10 amino acid medium containing 50 μCi of a mixture of 15 <sup>3</sup>H-labeled amino acids (TRK440; Amersham Buchler) and 5% dialyzed fetal bovine serum per ml. After another 15 min, this medium was removed, and the monolayer was washed twice with ice-cold phosphate-buffered saline and then lysed at room temperature with 0.5 ml of 50 mM Tris-hydrochloride, pH 6.8, containing 2% SDS, 4 M urea, and 2% β-mercaptoethanol per petri dish. The lysate was passed through a syringe needle 10 times, heated to 100°C for 2 min, and stored at -20°C.

**(ii) Tryptic peptide fingerprinting.** Cells were infected as described above (i). After 10 h of infection, the medium was replaced by medium without methionine or serum (minus methionine medium), and 15 min later this medium was replaced with minus methionine medium containing 100 μCi of [<sup>35</sup>S]methionine per ml and 5% dialyzed fetal bovine serum. After another 45 min, cell lysates were prepared as described above (i).

**Antisera and immunoprecipitation.** Antiserum against JHMV (anti-JHM serum) was produced in rabbits by three intramuscular injections of purified virus at 4-week intervals. Antiserum against viral protein p60 (anti-p60 serum) was prepared by two intraperitoneal injections of a homogenized polyacrylamide gel containing purified protein into specific-pathogen-

free NMRI mice. Preimmune sera were taken from animals which were shown to lack JHM-neutralizing antibodies. All sera were adsorbed to a powdered acetone extract of uninfected Sac(-) cells before use.

**Immunoprecipitation of the products of in vitro translation** was performed essentially as described by Paucha et al. (14), using a 1/100 dilution of antiserum or preimmune serum and adsorption to a suspension of protein A bearing *Staphylococcus aureus* (5).

**Preparation of RNA.** Poly(A) RNA was isolated from the cytoplasm of JHMV-infected or uninfected cells essentially as described by Wheeler et al. (25). Briefly, 5 × 10<sup>8</sup> Sac(-) cells in suspension were infected or mock infected as described above for virus purification. After 20 h of infection, the cells were pelleted and washed three times in Tris-buffered saline, pH 7.4. The cell pellet was suspended in 7 volumes of ice-cold 20 mM Tris-hydrochloride (pH 7.5)-100 mM NaCl-5 mM MgCl<sub>2</sub>. Resuspension of infected cells resulted in cell lysis, and the lysate was immediately centrifuged at 1,500 × g for 5 min at 4°C. With uninfected cells, it was necessary to add Triton X-100 to a final concentration of 0.1% to lyse the cells. Polyvinyl sulfate and SDS were added to the cytoplasmic lysate to final concentrations of 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. Poly(A) RNA was selected from this material by chromatography on polyuridylic acid-Sepharose (10).

<sup>3</sup>H-labeled RNA was prepared from Sac(-) cells infected in suspension at an MOI of 6 TCID<sub>50</sub>/cell. After 4 h of infection, the Spinner medium was replaced by Spinner medium containing twice the usual amount of vitamins and amino acids, 10% dialyzed fetal bovine serum, and 5 μg of actinomycin per ml, followed 30 min later by 30 μCi of [<sup>3</sup>H]uridine (TRK178; Amersham Buchler) per ml and 2.25 h later by 100 μg of cycloheximide per ml. At 6.75 h after infection, the cells were washed once in ice-cold TKM buffer (10 mM Tris-hydrochloride [pH 7.4], 150 mM KCl, 10 mM MgCl<sub>2</sub>), resuspended in TKM buffer containing 100 μg of polyvinyl sulfate per ml, and disrupted in a Dounce homogenizer. The lysate was centrifuged at 1,000 × g for 5 min at 4°C, and SDS was added to the supernatant to a concentration of 0.5%. RNA was extracted by repeated shaking with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Poly(A) RNA was selected by polyuridylic acid-Sepharose chromatography.

Polysomal RNA was prepared from Sac(-) cells infected and treated as described above, except the [<sup>3</sup>H]uridine was omitted and the washed cell pellet was suspended in TKM buffer containing 0.2% Nonidet P-40, 3.6% sucrose, 100 μg of cycloheximide per ml, 100 μg of polyvinyl sulfate per ml, and 0.05% β-mercaptoethanol. The 1,000 × g supernatant from the disrupted cell lysate was made 1% with sodium deoxycholate, layered onto a linear 15 to 40% sucrose gradient in TKMI buffer (TKM buffer containing 30 μg each of polyvinyl sulfate and cycloheximide per ml), and centrifuged for 2.5 h at 25,000 rpm at 4°C in an SW27 rotor. The region of the gradient containing polysomes sedimenting between about 100S and 300S, as judged by absorbance at 260 nm, was pooled, diluted

to a concentration of 15% sucrose with TKMI buffer, and overlaid onto a 5-ml cushion of 2 M sucrose in TKMI buffer. The polysomes were pelleted at 25,000 rpm for 12 h at 4°C in an SW27 rotor. The pellet was suspended in RSB buffer (10 mM Tris-hydrochloride [pH 7.4], 10 mM NaCl, 3 mM MgCl<sub>2</sub>), RNA was released from the polysomes by puromycin treatment, and ribosomal subunits were separated on a 5 to 20% sucrose gradient as described by Blobel and Sabatini (1). Material sedimenting slower than 50S was pooled and diluted with water to a sucrose concentration of 7.5%, and a 1/10 volume of 10% SDS, a 1/10 volume of 10× RNA extraction buffer (500 mM NaCH<sub>3</sub>COOH [pH 5.0], 1 M NaCl, 25 mM EDTA), and ascites tRNA to a concentration of 10 µg/ml were added before RNA was extracted by repeated shaking with phenol-chloroform-isoamyl alcohol. The RNA was recovered by alcohol precipitation, re-precipitated three times, and stored in water at -70°C.

**Cell-free protein-synthesizing systems.** The rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (15), except the amino acids (minus methionine) were added to a concentration of 0.2 mM each and 2-aminopurine was added to a final concentration of 6 mM. Incubation was for 1 h at 32°C. The efficiency of incorporation was determined as described by Dahl and Dickson (3). Samples for electrophoresis on SDS-polyacrylamide gels were prepared by mixing 1 µl of the reaction mixture with 9 µl of water and 10 µl of electrophoresis sample buffer, followed by heating to 100°C for 2 min.

The messenger-dependent L-cell S30 was prepared as described by Paucha et al. (14) and Dahl and Dickson (3). The determination of incorporation efficiency and the preparation of samples for electrophoresis were as described by Paucha et al. (14).

**Gel electrophoresis.** Samples were electrophoresed on 15% discontinuous SDS-polyacrylamide gels as described by Laemmli (6). Electrophoresis sample buffer contained 2% SDS, 10% glycerol, 0.001% bromophenol blue, 0.1 M dithiothreitol, and 62.5 mM Tris-hydrochloride, pH 6.8. The procedures for staining and drying the polyacrylamide gels and the exposure of autoradiographs have been described by Smith et al. (19). In some cases, gels were impregnated with PPO (2,5-diphenyloxazole) and subjected to fluorography at -80°C (9).

**Tryptic peptide fingerprinting.** Tryptic peptide fingerprinting was performed as described by Siddell (17). The oxidized, trypsin-digested peptides were separated on thin-layer cellulose plates by electrophoresis in pH 2.1 buffer and ascending chromatography in butanol-acetic acid-water-pyridine.

**Sucrose-formamide gradient centrifugation.** Cytoplasmic poly(A) RNA and RNA released from polysomes were fractionated on 5 to 20% sucrose gradients containing 50% formamide as described by Smith et al. (18), but with centrifugation in an SW41 rotor at 30,000 rpm for 18 to 20 h at 18°C. Samples were denatured in 60% formamide-6 mM Tris-hydrochloride (pH 7.5)-0.6 mM EDTA at 37°C for 10 min before centrifugation.

**RNA markers.** <sup>3</sup>H-labeled rRNA was prepared from uninfected L-cells, and <sup>32</sup>P-labeled 18S rRNA was prepared from uninfected CV1 cells and was a gift

from J. R. Stephenson. Poly(A) RNA was removed from rRNA by two cycles of polyuridylic acid-Sepharose chromatography.

## RESULTS

**Protein synthesis in infected cells.** The [<sup>35</sup>S]methionine-labeled polypeptides of JHMV are shown in Fig. 1 (track V). The nomenclature used is based on the previously described molecular weights (hereafter K will be used to denote molecular weight × 1,000) of the proteins and whether they are glycosylated (22). The pattern is essentially as described by Wege et al., with some minor differences (S. G. Siddell, manuscript in preparation). The major glycoproteins of the virus are gp170, gp98, gp65, and gp25. Structural proteins p60 and p23 are not glycosylated, and p60 is the major nucleocapsid protein of the virus (22).

The infection of Sac(-) cells with a high multiplicity of JHMV led to an almost complete shutoff of host cell protein synthesis within 8 to 9 h (Fig. 1). Pulse-labeling the cells with a mixture of 15 <sup>3</sup>H-amino acids showed that from 5 h postinfection onward the major protein synthesized in infected cells was a 60K polypeptide which comigrated with the virion protein p60. At 7 to 8 h after infection, the specific synthesis

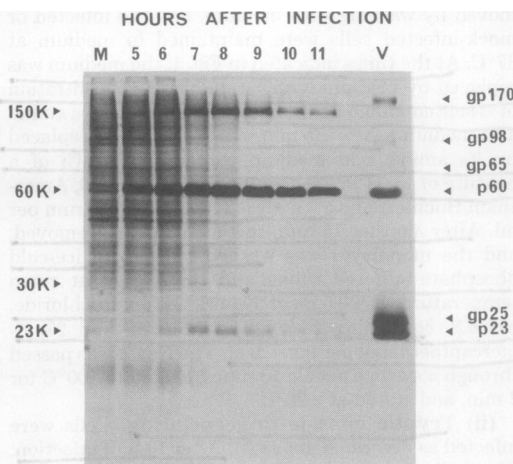


FIG. 1. Time course of JHMV-specific protein synthesis. Sac(-) cells were infected with JHMV and pulse-labeled with a mixture of <sup>3</sup>H-amino acids at the times indicated, and lysates were prepared as described in the text. Samples of each lysate were mixed with equal volumes of electrophoresis sample buffer, heated to 100°C for 2 min, and electrophoresed in parallel with a sample of purified virus. JHMV was labeled with [<sup>35</sup>S]methionine and purified as described in the text. Labeled virus was mixed with an equal volume of electrophoresis sample buffer and heated to 37°C for 2 min before electrophoresis. The gel was fluorographed.

in infected cells of two more major polypeptides (150K and 23K) and a fourth minor polypeptide (30K) was also detected. Of these, only the 23K protein comigrated with a virion protein, i.e., p23.

Confirmation that the 60K intracellular protein had a primary structure similar, if not identical, to p60 was provided by tryptic peptide fingerprinting. Figure 2A and D shows that p60 from the virus and the putative p60 from the cell

both had six major [<sup>35</sup>S]methionine-containing tryptic peptides, five of which comigrated in chromatography and electrophoresis. Although the peptide labeled X migrated differently when the samples derived from the virion or the cell were electrophoresed separately, a sample containing equal radioactive amounts of digest from each protein produced a fingerprint in which all six peptides comigrated (Fig. 2C). We cannot yet explain the apparent variable electrophoretic

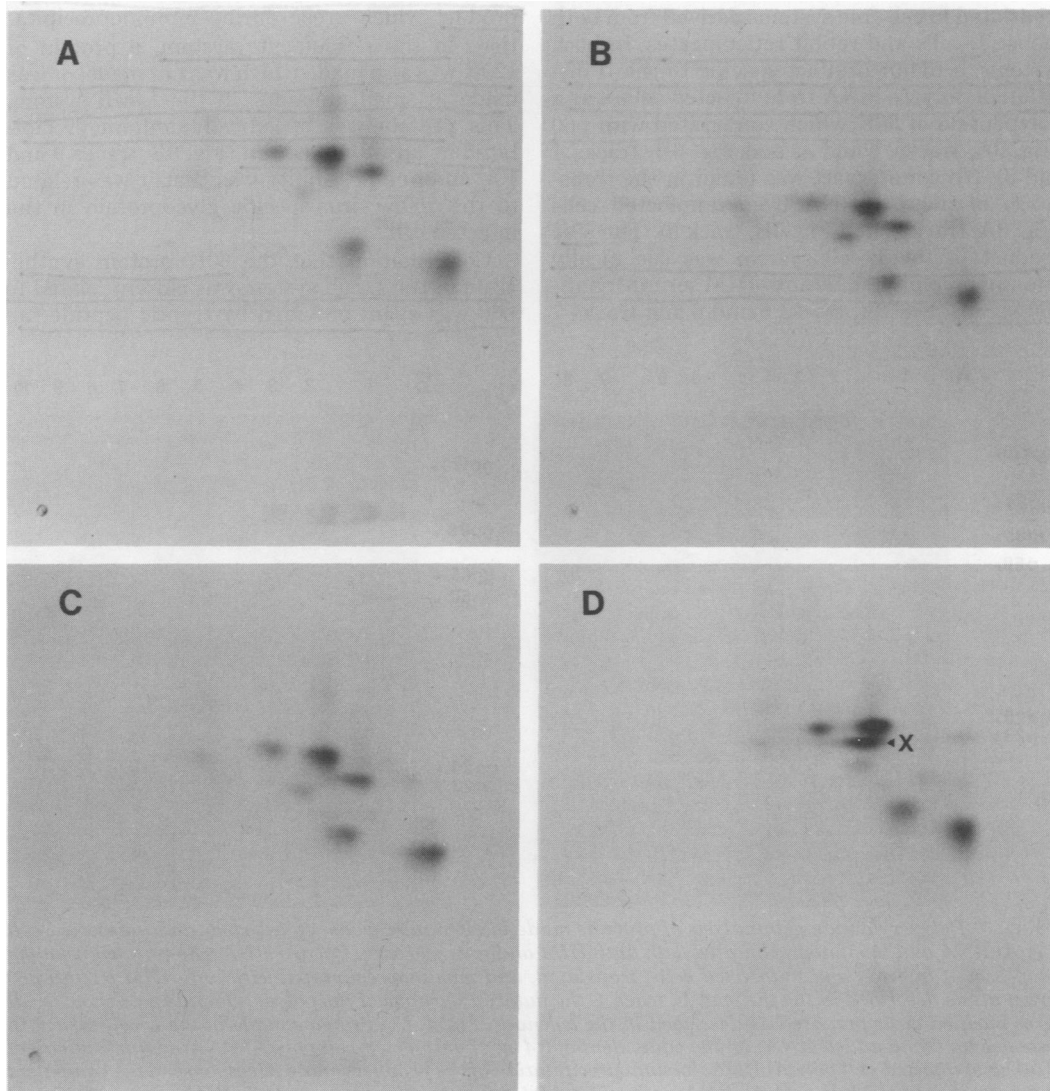


FIG. 2. Tryptic peptide fingerprints of JHMV p60 made *in vitro*, purified virion p60, and p60 synthesized in infected cells. Samples of [<sup>35</sup>S]methionine-labeled virus, [<sup>35</sup>S]methionine-pulse-labeled cell lysate, and [<sup>35</sup>S]methionine-labeled L-cell translation products were prepared and electrophoresed, the area of the gel containing p60 was cut out, and the protein was eluted and processed all as described in the text. Electrophoresis was from left to right, and chromatography was from bottom to top. (A) p60 from purified JHMV; (B) p60 made *in vitro*; (C) a mixture of equal radioactive amounts (25,000 cpm) of (A), (B), and (D); (D) p60 purified from infected cells.

mobility of peptide X, but nevertheless we conclude from these data that the two proteins are closely related, if not identical. The identity of the 23K intracellular protein as p23 has also been confirmed by specific immunoprecipitation with anti-JHM serum and tryptic peptide fingerprinting (S. G. Siddell and H. Wege, manuscript in preparation).

**In vitro translation of infected-cell poly(A) RNA.** Poly(A) RNA isolated from the cytoplasm of infected or mock-infected cells was translated in cell-free systems derived from both mouse L-cells and rabbit reticulocytes. In both systems, a major product specific to the translation of poly(A) RNA from infected cells was a polypeptide of 60K which comigrated with p60 (Fig. 3A, tracks 1 and 4, and Fig. 3B, tracks 1 and 8). No counterpart was found in the translation of poly(A) RNA from uninfected cells (Fig. 3A, track 3, and Fig. 3B, track 5). The 60K product of the L-cell system was specifically immunoprecipitated by anti-JHM serum or anti-p60 serum (Fig. 3A, tracks 5 and 6 and tracks 7

and 8), and no such protein was immunoprecipitated from the translation of uninfected-cell poly(A) RNA (data not shown).

The 60K product of the reticulocyte system was also specifically immunoprecipitated by anti-JHM serum (Fig. 3B, tracks 9 and 10). The immunoprecipitate of the reticulocyte translation (Fig. 3B, track 9) clearly contained a major band migrating just ahead of the 60K product which was not present in the translation. We assumed that this protein was a degradation product which arose during immunoprecipitation. In the reticulocyte system, a protein of 120K was also made which was not made, or was made in small amounts, in the L-cell system. This protein was specifically immunoprecipitated by anti-JHM serum (Fig. 3B, tracks 9 and 10), and one possibility was that it was related to the 150K virus-specific glycoprotein in the infected cell.

Confirmation that the 60K protein synthesized in the L-cell system was closely related to p60 was again provided by tryptic peptide fin-

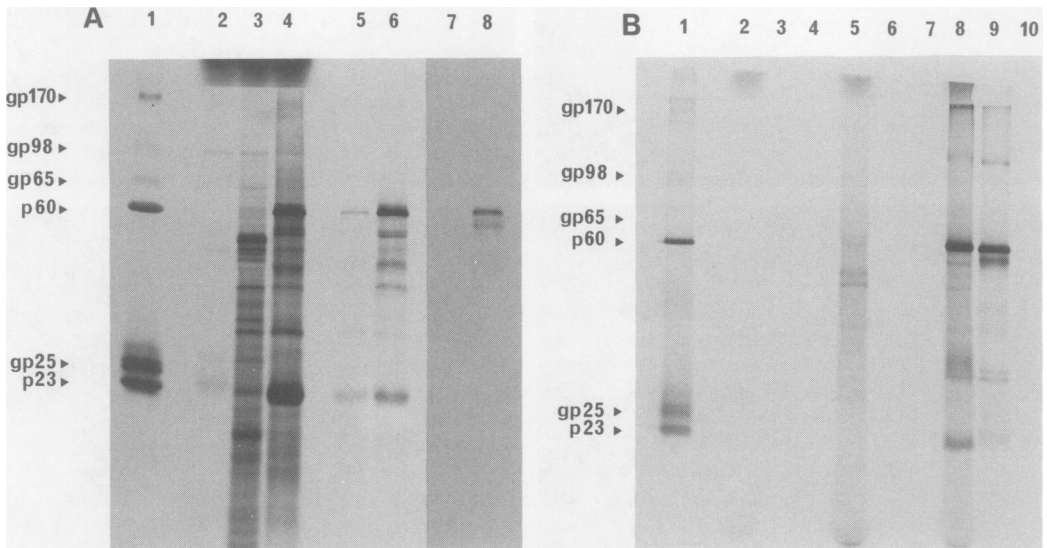


FIG. 3. Polyacrylamide gel analysis of proteins made *in vitro* in response to infected- and uninfected-cell poly(A) RNA and immunoprecipitated with anti-JHM and anti-p60 sera. Poly(A) RNA was isolated from the cytoplasm of infected and uninfected cells, translated, and immunoprecipitated with anti-JHM or anti-p60 serum all as described in the text. In track 1, the autoradiographs of the polyacrylamide gels show in all cases purified virus prepared as described in the legend to Fig. 1. (A) Proteins made in the L-cell system in response to: (2) no added RNA; (3) the equivalent of 0.4  $\mu$ g of uninfected-cell poly(A) RNA; (4) the equivalent of 0.4  $\mu$ g of infected-cell poly(A) RNA. Immunoprecipitation of the products made in response to the equivalent of 0.4  $\mu$ g of infected-cell poly(A) RNA with: (5) rabbit preimmune serum; (6) anti-JHM serum; (7) mouse preimmune serum; (8) anti-p60 serum. (B) Proteins made in the reticulocyte system in response to: (2) no added RNA; (3) the equivalent of 40 ng of uninfected-cell poly(A) RNA; (4) the equivalent of 40 ng of infected-cell poly(A) RNA. Immunoprecipitation with rabbit preimmune serum of the products made in response to: (5) no added RNA; (6) the equivalent of 200 ng of uninfected-cell poly(A) RNA; (7) the equivalent of 200 ng of infected-cell poly(A) RNA. Immunoprecipitation with anti-JHM serum of the products made in response to: (8) no added RNA; (9) the equivalent of 200 ng of uninfected-cell poly(A) RNA; (10) the equivalent of 200 ng of infected-cell poly(A) RNA.

gerprinting. Figure 2A, B, and C shows that the [ $^{35}\text{S}$ ]methionine-containing tryptic peptides of the putative p60 made in vitro were identical to those of virion p60 when electrophoresed and chromatographed separately or in combination. By the criteria of comigration, specific immunoprecipitation, and peptide fingerprinting, we concluded that the protein made in vitro was p60.

In both cell-free systems, many more polypeptides were synthesized in response to infected-cell poly(A) RNA. Among these was a 23K polypeptide which was a major product of the L-cell system, comigrated with virion protein p23, was specifically immunoprecipitated by anti-JHM serum, and had a [ $^{35}\text{S}$ ]methionine-containing tryptic peptide fingerprint similar, if not identical, to that of virion p23 (Fig. 3A, tracks 1, 5, and 6; unpublished data). We concluded that this product was p23. The apparent discrepancy between the amounts of p23 synthesized in the infected cell and those synthesized in response to cytoplasmic poly(A) RNA in vitro (compare Fig. 1 and 3A) has not been explained, but may be accounted for by a larger number of methionine residues in p23 than is normal. We are currently investigating this possibility.

To establish the size of the poly(A) RNAs that encode p60 and p23, we first investigated the poly(A) RNA synthesized in the cytoplasm of actinomycin D-treated cells late in infection. Figure 4 shows the sedimentation in a sucrose-formamide gradient of cytoplasmic poly(A) RNA, pulse-labeled in the presence of actinomycin D, in combination with  $^{32}\text{P}$ -labeled 18S rRNA. The major species synthesized within the size range analyzed in this gradient sedimented at 17S. A poly(A)-containing RNA with the same sedimentation value was also a major species specifically released by puromycin or EDTA treatment of polysomes isolated at the same time of infection (unpublished data). With sucrose-formamide gradients, it was not possible to clearly resolve additional species in this size range, although we believe that the shoulder at 19S indicates another RNA species.

Second, we sedimented poly(A) RNA from the cytoplasm of infected cells on sucrose-formamide gradients, fractionated the gradient, recovered the RNA from each fraction, and translated it in vitro. Figure 5A shows the products made with unfractionated RNA (track 2) and selected fractions of the same material after centrifugation (tracks 3 to 14). The translation products of the portion of the gradient including RNA sedimenting at 15S to 25S, as judged by the sedimentation of L-cell [ $^3\text{H}$ ]rRNA in a parallel gradient, are shown. The RNA encoding the 60K product sedimented at about 17S, and

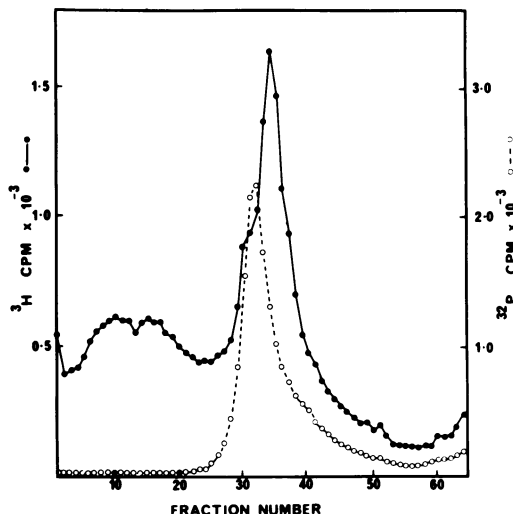


FIG. 4. Sedimentation of cytoplasmic poly(A) RNA synthesized in actinomycin D-treated cells late in infection. A total of 75,000 cpm of [ $^3\text{H}$ ]uridine-labeled cytoplasmic poly(A) RNA was prepared as described in the text and cosedimented in a sucrose-formamide gradient with 25,000 cpm of  $^{32}\text{P}$ -labeled 18S Vero cell rRNA. Symbols: (●)  $^3\text{H}$ -labeled RNA; (○)  $^{32}\text{P}$ -labeled 18S rRNA. Sedimentation was from right to left.

the protein could be identified as p60 both by comigration with virion p60 (Fig. 5A and B) and by immunoprecipitation with anti-p60 serum (Fig. 5B). There was no immunoprecipitation with preimmune serum (data not shown). A more accurate sedimentation value of 17S was obtained from the translation of poly(A) RNA fractionated in combination with rRNA markers (data not shown). We have analyzed gradients which would include RNA sedimenting up to 50S and have not found another cytoplasmic poly(A) RNA species which directs the synthesis of p60 in vitro.

Figure 5 also shows that size-fractionated poly(A) RNA directed the synthesis of other proteins in vitro. A series of these was specifically immunoprecipitated by anti-p60 serum, and the RNA which encoded them had the same size distribution as that encoding p60. These products may be premature terminations of the in vitro system which were related to p60. It is unlikely that they were cleavage products of p60 as they were synthesized in vitro in the presence of 600 Kallikrein units of aprotinin, a protease inhibitor, per ml (data not shown).

Another major translation product of fractionated poly(A) RNA of this size class was a 23K protein which comigrated with virion protein p23. This product was not immunoprecipitated with anti-p60 serum (Fig. 5B; although a precipitate is visible in track 10, compare the relative

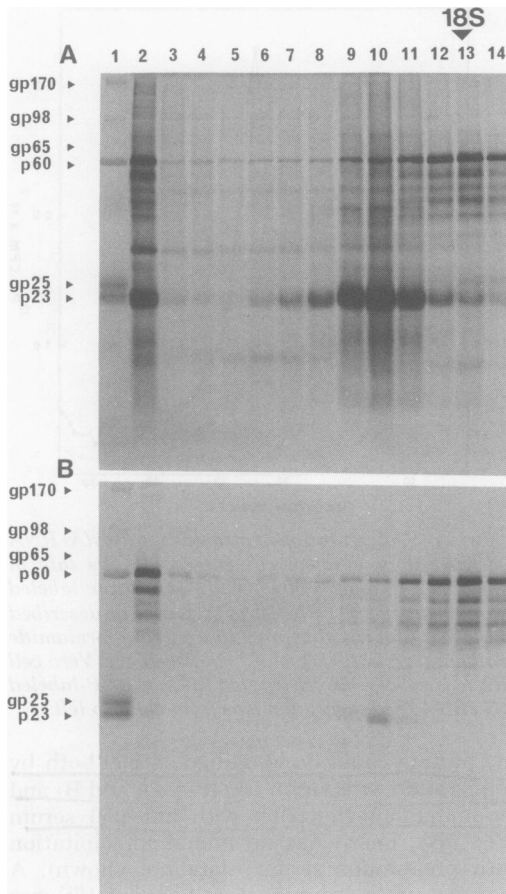


FIG. 5. *In vitro* translation of fractionated poly(A) RNA from JHMV-infected cells and immunoprecipitation of the products with anti-p60 serum. Poly(A) RNA was isolated from JHMV-infected cells as described in the text. RNA (20  $\mu$ g) was denatured and sedimented on a sucrose-formamide gradient. Fifty-eight fractions were collected, and the RNA in each fraction was recovered, precipitated twice, and suspended in 15  $\mu$ l of water. Samples were translated in the L-cell system. Purified virus prepared as described in the legend to Fig. 1 is shown in track 1. (A) Products made in response to: (2) the equivalent of 0.33  $\mu$ g of unfractionated RNA; (3 to 14) 2  $\mu$ l of RNA recovered from fractions 19 to 31, respectively. (B) (2 to 14) Translation products identical to (2) to (14) in (A) which were immunoprecipitated with anti-p60 serum. Sedimentation is from right to left, and  $^3$ H-labeled L-cell rRNA was analyzed in a parallel gradient. The 4S, 18S, and 28S rRNA markers sedimented in fractions corresponding to 49, 28, and 12, respectively.

immunoprecipitation of p60 and the precipitation of the 23K product in tracks 2, 10, and 13), but was immunoprecipitated by anti-JHM serum (data not shown; Fig. 3). The RNA activity encoding the 23K product sedimented at about

19S and was clearly separable from that encoding p60.

In vitro translation of infected-cell mRNA. We have presented data on the *in vitro* translation products and the sizes of poly(A) RNA isolated from the cytoplasm of infected cells. To confirm that these RNAs were mRNA's in the cell, we also investigated the sizes and translation products of RNAs which were specifically released from infected-cell polysomes in such a way as to exclude contamination with RNA derived from nucleocapsids which might have been present in the polysome material (see above). The results that we obtained were essentially identical to those obtained with cytoplasmic poly(A) RNA. Infected-cell mRNA directed the synthesis in the L-cell system of a similar spectrum of proteins as did cytoplasmic poly(A) RNA, and among the products were proteins which comigrated with p60 and p23. Both products were specifically immunoprecipitated with anti-JHM serum, whereas only the 60K product was specifically immunoprecipitated with anti-p60 serum.

We also confirmed the sizes of the mRNA's which encoded these proteins, again by fractionation on sucrose-formamide gradients. The *in vitro* translation of fractionated mRNA again indicated that the mRNA's encoding p60 and p23 sedimented at 17S and 19S, respectively, and were clearly separable.

## DISCUSSION

The data presented in this paper provide some of the first information on coronavirus replication. At late times of infection, the major protein synthesized in JHMV-infected cells was shown to be one of the virion nucleocapsid proteins, p60. Also synthesized in infected cells, although in lesser amounts, was a 23K polypeptide which was identified as virion protein p23. The synthesis of these proteins was easily detected as host cell protein synthesis was efficiently shut off in JHMV-infected Sac(-) cells, an apparent discrepancy with earlier reports (2) that is probably accounted for by the higher MOI used in our study.

The poly(A) RNAs encoding p60 and p23 were isolated, translated *in vitro*, and found to sediment in sucrose-formamide gradients at 17S and 19S, respectively. Both of the RNAs could be specifically released from infected-cell polysomes and are therefore physiological mRNA's. Also, the 17S and, we believe, the 19S RNAs constitute a large proportion of the viral RNA synthesized late in infection and are therefore unlikely to result from degradation. Our interpretation of these data is that these RNAs represent two subgenomic coronavirus JHM

mRNA's, each of which encodes a different structural protein of the virus.

The data do not, however, exclude a number of more complex interpretations. For example, the mRNA's which sediment at 17S and 19S could be conformational forms, one of which has an active internal initiation site. Although indirect, evidence for internal initiation of positive-stranded virus mRNA has been reported (24), and the sizing of mRNA on sucrose-formamide gradients probably depends to a degree on the hydrodynamic properties of the RNA. Possibilities such as this can only be excluded by analysis of the mRNA's themselves.

If our interpretation is correct, the replication strategy of JHMV contrasts with other well-characterized nontransforming positive-stranded RNA animal viruses. Unlike picornaviruses, alphaviruses, and flaviviruses, the expression of the JHMV genome appears to involve more than one subgenomic mRNA, at least one of which is apparently monocistronic (i.e., the 17S RNA encodes a 60K protein) and one of which clearly has a potential coding capacity in excess of that required to encode its translation product (i.e., the 19S RNA encodes a 23K protein). Our studies on protein synthesis in the infected cell and on the *in vitro* translation and characterization of mRNA's which encode virus-specific proteins indicate that in addition to these two mRNA's, at least another one, and possibly more subgenomic mRNA's, is involved in the replication strategy of coronaviruses, in addition to the assumed mRNA activity of the genomic RNA. Our future experiments will be aimed at defining these RNAs, their products, and their relationships to each other, eventually arriving at an understanding of the molecular biology of JHMV.

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#### LITERATURE CITED

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