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## Isolation and Identification of Virus-Specific mRNAs in Cells Infected with Mouse Hepatitis Virus (MHV-A59)

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We have determined the kinetics of virus production and virus-specific RNA synthesis in Sac(-) cells infected with mouse hepatitis virus strain A59 (MHV-A59). Immunofluorescence showed that all cells became infected at a multiplicity of 10 PFU/cell. The virus was concentrated and purified to obtain the high titered stocks needed for these one-step growth experiments. Release of virus into the culture medium started 4 hr after infection (pi) and was complete at 10 hr pi. Synthesis of virus-specific RNA, measured by the incorporation of [<sup>3</sup>H]uridine in the presence of 1 μg/ml actinomycin D, also started at 4 hr pi and its maximum rate occurred between 6 and 8 hr pi. RNA labeled during this period was isolated from infected cells. About 50% of this RNA bound to oligo(dT)-cellulose; this material was denatured with glyoxal-dimethyl sulfoxide and analyzed by electrophoresis in 1% agarose gels. Seven RNA species with the following molecular weights were present: 5.6 × 10<sup>6</sup> (RNA1), 4.0 × 10<sup>6</sup> (RNA2), 3.0 × 10<sup>6</sup> (RNA3), 1.4 × 10<sup>6</sup> (RNA4), 1.2 × 10<sup>6</sup> (RNA5), 0.9 × 10<sup>6</sup> (RNA6), and 0.6 × 10<sup>6</sup> (RNA7). RNA1 comigrated with the viral genome. Artifacts caused by defective interfering particles or breakdown of RNA were excluded. To determine whether these RNA species were functional as messengers in infected cells, virus-specific RNAs present in polyribosomes were analyzed. EDTA treatment was used to discriminate between RNA present in polyribosomes and in EDTA-resistant, presumably ribonucleoprotein, particles. Most (91%) of RNA1 was present in EDTA-resistant particles; the remainder and all other RNAs synthesized between 6 and 8 hr pi were present in polyribosomes. We conclude that MHV-A59 has six subgenomic mRNAs. Since the total molecular mass (11.1 × 10<sup>6</sup> daltons) of these messengers is about twice that of the viral genome, sequence homologies must exist between the mRNAs. The position of these homologous regions and the translation products of each of the mRNAs remain to be determined.

### INTRODUCTION

Ten strains of mouse hepatitis virus (MHV) have been isolated so far (MHV-1, Gledhill and Andrewes, 1951; MHV-2, Nelson, 1952; MHV-3, Dick *et al.*, 1956; MHV-4(JHM), Cheever *et al.*, 1949; MHV-A59, Manaker *et al.*, 1961; MHV-S/CDC, Hierholzer *et al.*, 1979; MHV-S, Rowe *et al.*, 1963; MHV-LS, Sabesin, 1972; H747, Nelson, 1952; EHF 210, Nelson, 1952). These viruses were grouped together on the basis of their antigenic relationship. They usually cause fatal hepatitis but if the acute

effects of the infection are suppressed, with the use of *ts*-mutants of the virus or of hosts with a genetic resistance against MHV, strains MHV-3, MHV-4, and MHV-A59 can cause demyelination of the central nervous system or other chronic neurological diseases (Lampert *et al.*, 1973; Weiner, 1973; Virelizier *et al.*, 1975; Herndon *et al.*, 1975, 1977; Haspel *et al.*, 1978; Nagashima *et al.*, 1978, 1979; Robb *et al.*, 1979). There are no data as yet to explain these neurological disorders at the molecular level. This is due, in part, to the lack of information about the replication mechanism of MHV and other members of the coronavirus family.

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Coronavirions are about 100 nm in diameter and possess characteristic club-shaped surface projections 20 nm in length (McIntosh, 1974; Tyrrell *et al.*, 1975, 1978). The infectious genome of MHV consists of a linear single-stranded RNA, with an apparent molecular weight of about  $6 \times 10^6$  (Lai and Stohlman, 1978; Wege *et al.*, 1978). Consequently these viruses have the largest genome of the class IV viruses in Baltimore's classification system (Baltimore, 1971). The number of structural polypeptides reported for mouse hepatitis virus varies from six proteins for MHV-4 (Wege *et al.*, 1979) to four proteins for MHV-A59 (Sturman, 1977).

Two subclasses of positive-stranded class IV RNA viruses can be distinguished, depending on how many forms of mRNA are present in infected cells. One subclass, the picornaviruses, synthesizes one mRNA which has the same length as genomic RNA. The structural proteins are formed by proteolytic cleavage of a polyprotein (Sangar, 1979). The other subclass, the alphaviruses, uses two forms of mRNA. One has the same size as virion RNA, the other is a subgenomic mRNA, representing the 3' portion of the genome (Kääriäinen and Söderlund, 1978).

There is suggestive evidence for subgenomic RNA(s) in coronavirus-infected cells (Robb and Bond, 1979). Our aim was to investigate how many mRNA species are involved in the replication of the MHV strain A59. In this paper we provide evidence that there is a third subclass of positive-stranded RNA viruses because multiple mRNA species were found in cells infected with MHV-A59.

#### MATERIALS AND METHODS

*Cells and virus.* Mouse L cells and Sac(-) cells, a Moloney sarcoma virus-transformed cell line defective in virus production (Weiland *et al.*, 1978), were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (DMEM-10% FCS) containing penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml).

The A59 strain of mouse hepatitis virus (Manaker *et al.*, 1961), obtained from

the American Type Culture Collection (Rockville, Md.), was plaque-purified twice before virus stocks were prepared by infecting Sac(-) cells at a multiplicity of infection (m.o.i.) of 0.2. Virus titers of  $2-5 \times 10^7$  PFU/ml were usually obtained. This virus was used to obtain high-titered virus stocks needed for infection of cells at a high m.o.i. Concentrated virus was used in all experiments; it was prepared as described below.

Unless otherwise indicated Sac(-) cells were infected at a density of  $1-2 \times 10^6$  cells/35-mm tissue culture dish ( $1-2 \times 10^7$  cells/75 cm<sup>2</sup> tissue culture flask) using 10 PFU/cell. Inocula were in 0.3 ml (1.5 ml) DMEM-3% FCS. They were replaced by fresh DMEM-10% FCS after adsorption for 1 hr at 37°.

The virus was plaque titrated on L cells. An overlay of 1.5% Bacto-agar (Difco, Detroit, Mich.) in DMEM-1% FCS was used. After 3 days of incubation the plates were stained with 0.02% neutral red in phosphate-buffered saline (PBS) and plaques counted.

*Purification of virus.* Virus obtained from roller bottle cultures infected at 2 PFU/cell was precipitated from clarified infectious culture medium by the addition of 100 g/liter polyethylene glycol 6000 (BDH, Poole, England) and 23.3 g/liter NaCl, pH 7.7. After 2 hr of incubation at 4° the virus suspension was centrifuged at 10,000 *g* for 15 min at 4°. The pellet was resuspended in a small volume of TES-buffer (0.02 *M* Tris-hydrochloride, 1 *mM* EDTA, 0.1 *M* NaCl, pH 7.4) and clarified (5 min, 10,000 *g*). This suspension was layered onto 16 ml 10% (w/w) sucrose in TES-buffer on top of a 16 ml linear 20-50% (w/w) sucrose gradient and centrifuged for 6 hr at 24,000 rpm in an SW 27.0 or SW 27.1 rotor. The light-scattering virus band at a density of 1.18 g/ml was collected. The overall recovery of infectivity was 60%. Band material negatively stained for electron microscopy contained typical coronavirus like particles.

*Antisera and immunofluorescence assay.* To obtain anti-MHV-A59 serum, 8-week-old BALB/c mice kept in isolators were infected intraperitoneally with 100 PFU. All mice became sick and about 50% died.

Survivors were bled 1 month after infection. The serum had a titer of 2000 in a plaque reduction assay (50% reduction of 100 PFU). Presera were negative. FITC-labeled goat anti-mouse IgG was purchased from Miles-Yeda Ltd. Rehovot, Israel. Both sera were used (dilution 1:20 and 1:30, respectively) in an immunofluorescence assay on acetone-fixed Sac(-) cells to establish the infected fraction as a function of the m.o.i.

*Kinetics of labeling of viral RNA.* Sac(-) cells, grown on 18 × 18 mm coverslips in 35-mm tissue culture dishes, were infected or mock-infected. After adsorption 1 ml DMEM-10% FCS containing 1 μg/ml of actinomycin D (act D) was added. Two hours after infection (pi) 10 μCi of [5-<sup>3</sup>H]-uridine (27.8 Ci/mmol; The Radiochemical Centre, Amersham, England) was added and incubation at 37° was continued. At time intervals the incorporation of [<sup>3</sup>H]uridine was measured in the following way: the coverslips were washed once in PBS, twice in 5% trichloroacetic acid, and once in ether-ethanol. The coverslips were placed in vials containing scintillation fluid and the amount of radioactivity was determined.

*Isolation of [<sup>3</sup>H]uridine-labeled RNA from infected cells and virus.* Monolayer cultures (approximately 10<sup>7</sup> cells/culture) were infected as previously described. Five hours pi the culture fluid was replaced with 6 ml DMEM-10% FCS containing 1 μg/ml of act D and 1 hr later 25 μCi/ml [<sup>3</sup>H]-uridine was added. After an incubation period of a further 2 hr the monolayers were washed once with ice-cold PBS. All subsequent operations were performed between 0 and 4°. The cells were lysed with 2 ml TES-buffer containing 0.5% Triton X-100 and 0.5% 1,5-naphthalenedisulfonate·Na<sub>2</sub>. The extract was centrifuged at 1000 g for 5 min and an equal volume of TES-buffer containing 2% SDS and 7 M urea (Aristar grade, BDH, Poole, England) was added to the post-nuclear supernatant. The RNA was phenol-extracted and ethanol-precipitated as described below. To prepare [<sup>3</sup>H]uridine-labeled virus, cells were labeled with 50 μCi/ml in DMEM-10% FCS from 3 to 10 hr pi. Virus-containing medium was harvested at 10 hr pi, precipitated with

polyethylene glycol 6000, and purified in sucrose gradients as previously described. Sucrose gradient fractions containing virus were diluted with an equal volume of TES-buffer and then disrupted by adding an equal volume of TES-buffer containing 1% SDS and 0.4 mg/ml proteinase K; the mixture was incubated for 5 min at 50° and then for a further 30 min at 25°. The RNA was phenol-extracted and ethanol-precipitated.

*Isolation of MHV-A59-specific polyosomes.* Monolayer cultures of Sac(-) cells in 75-cm<sup>2</sup> T-flasks were infected and labeled with [<sup>3</sup>H]uridine as previously described for the isolation of total RNA. Cycloheximide (100 μg/ml) was added 15 min before the cells were harvested at 8 hr pi. At this time the flasks were immersed in an ice-water slurry and after the cells had been washed with ice-cold PBS containing 100 μg/ml of cycloheximide they were lysed with 2 ml lysis buffer (20 mM Tris-hydrochloride, pH 7.5, 240 mM KCl, 7.5 mM MgCl<sub>2</sub>, 1% Triton X-100, 1 mM dithiothreitol, and 1 μg/ml cycloheximide). After 5 min incubation at 4° nuclei and cell debris were removed by centrifugation at 1000 g for 5 min at 4°. The polyosomes and other cytoplasmic material present in the postnuclear supernatant were analyzed by sedimentation of an 0.2 ml sample in a 4.5 ml isokinetic 15 to 32% (w/w) sucrose gradient (Van der Zeijst and Bloemers, 1976) constructed on top of an 0.5 ml sucrose cushion (50% w/w); the sucrose solutions were made up in gradient buffer (50 mM Tris-hydrochloride, pH 7.5, 80 mM KCl, 5 mM MgCl<sub>2</sub>, 7 mM 2-mercaptoethanol, and 0.1% Triton X-100). The gradient was centrifuged for 25 min at 50,000 rpm and 5° in an SW50.1 rotor and fractions were collected. An equal volume of gradient buffer supplemented with 5% (w/v) SDS, 0.5% 1,5-naphthalenedisulfonate·Na<sub>2</sub>, and 2.5 M urea was added to each fraction. A sample from each fraction was precipitated with trichloroacetic acid on Whatman 3 MM paper and the amount of radioactivity was counted. The remaining part of the RNA containing fractions was phenol-extracted and ethanol-precipitated after the addition of 20 μg/ml *Escherichia coli* rRNA. Poly-

ribosomes were dissociated by the addition of EDTA (final concentration 20 mM) to the postnuclear supernatant.

**Phenol extraction.** An equal volume of redistilled phenol, saturated with TES-buffer and containing 0.1% 8-hydroxyquinoline, was added to the sample at room temperature. After centrifugation RNA was precipitated from the aqueous phase by the addition of 2 volumes of ethanol and of 1 M sodium acetate pH 5.2 to a final concentration of 0.1 M. The mixture was kept at  $-20^{\circ}$  for at least 16 hr and the RNA was recovered by centrifugation at 10,000 *g* for 20 min at  $0^{\circ}$ . Carrier rRNA from *E. coli* (strain MRE600) was isolated from polyribosomes prepared as described by Godson and Sinsheimer (1967).

**Oligo(dT)-cellulose column chromatography.** Thirty milligrams of oligo(dT)-cellulose type 7 (P-L Biochemicals Inc., Milwaukee, Wisc.) was added to a 1-ml disposable syringe and prewashed with high salt binding buffer (10 mM Tris-hydrochloride, pH 7.5, 0.05% SDS, 0.5 M NaCl). The RNA was dissolved in the same buffer and applied to the column. Poly(A)-containing RNA was eluted with 10 mM Tris-hydrochloride, pH 7.5 and recovered by ethanol-precipitation after addition of *E. coli* rRNA (20  $\mu$ g/ml).

**Agarose gel electrophoresis of RNA.** RNA

was denatured with glyoxal and dimethyl sulfoxide ( $\text{Me}_2\text{SO}$ ) using a modification of the method described by McMaster and Carmichael (1977). RNA was dissolved in denaturation buffer (10 mM sodium phosphate buffer, pH 7.0, 1 M deionized glyoxal, 50% (v/v)  $\text{Me}_2\text{SO}$ , 1 mM EDTA, and 0.25% SDS) and heated for 30 min at  $56^{\circ}$ . A horizontal 1% agarose slabgel (19  $\times$  19  $\times$  0.2 cm; Agarose C, Pharmacia Fine Chemicals, Uppsala, Sweden) in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, 33 mM acetic acid, 2 mM EDTA, pH 7.4) supplemented with 6 M deionized urea was used. The gel was cooled to  $5^{\circ}$  during electrophoresis (5–6 hr at 150 V). In one experiment (Fig. 2A) urea was omitted from the gel; 10 mM sodium phosphate pH 7.0 was used as buffer. In this case electrophoresis was at 200 V for 3.5 hr. After electrophoresis the gel was fixed in methanol, then soaked for 2 hr in EN<sup>3</sup>HANCE (New England Nuclear, Dreieich, West Germany) and dried under vacuum at room temperature for 5 min. The enhancing material was precipitated inside the gel by the addition of water and the gel was kept in water for 30 min before it was dried under vacuum at  $80^{\circ}$ . Fluorography was performed by exposure of the dried gel to a preflashed Kodak XR film at  $-70^{\circ}$  (Laskey and Mills, 1975).

TABLE 1

PERCENTAGE OF SAC(-) CELLS INFECTED WITH MHV-A59 AS A FUNCTION OF THE MULTIPLICITY OF INFECTION (M.O.I.)<sup>a</sup>

m.o.i. (PFU/cell)	Percentage infected	
	Experiment	Theory
0	0	0
0.1	12	10
0.5	37	39
1.0	65	63
5.0	100	99
10.0	100	100

<sup>a</sup> Percentages of infected Sac(-) cells were determined 8 hr pi by immunofluorescence; theoretical values were calculated from the Poisson distribution, using the virus titers obtained by plaque titration on L cells.

## RESULTS

*Virus Growth*

Infection of murine cells with MHV may induce the production of endogenous retroviruses (Wege *et al.*, 1978). Sac(-) cells were chosen as host cells for MHV-A59 since these cells are defective in the synthesis of retroviral proteins (Weiland *et al.*, 1979). This cell line does not form confluent monolayers and is therefore less suitable for plaque-titration; instead L cells were employed, with highly reproducible results. Table 1 shows that MHV-A59 infects mouse L cells and Sac(-) cells equally well, since the percentage of infected Sac(-) cells as a function of the m.o.i. calculated from the virus titers obtained by plaque-titration on L cells closely followed the

theoretical values expected according to the Poisson distribution.

We wanted to study the replication of MHV-A59 under single cycle conditions. At a virus concentration of 10 PFU/cell every cell became infected, so we have used this m.o.i. in all further experiments.

The kinetics of virus release into the

medium are shown in Fig. 1A. Virus production was complete at 10 hr pi by which time the cells had formed syncytia. About 50% of the infectivity remained cell-bound at 10 hr pi. Since we wished to use act D in most experiments, virus growth in cells incubated with 1  $\mu\text{g/ml}$  act D and untreated cells was compared. There was no signifi-

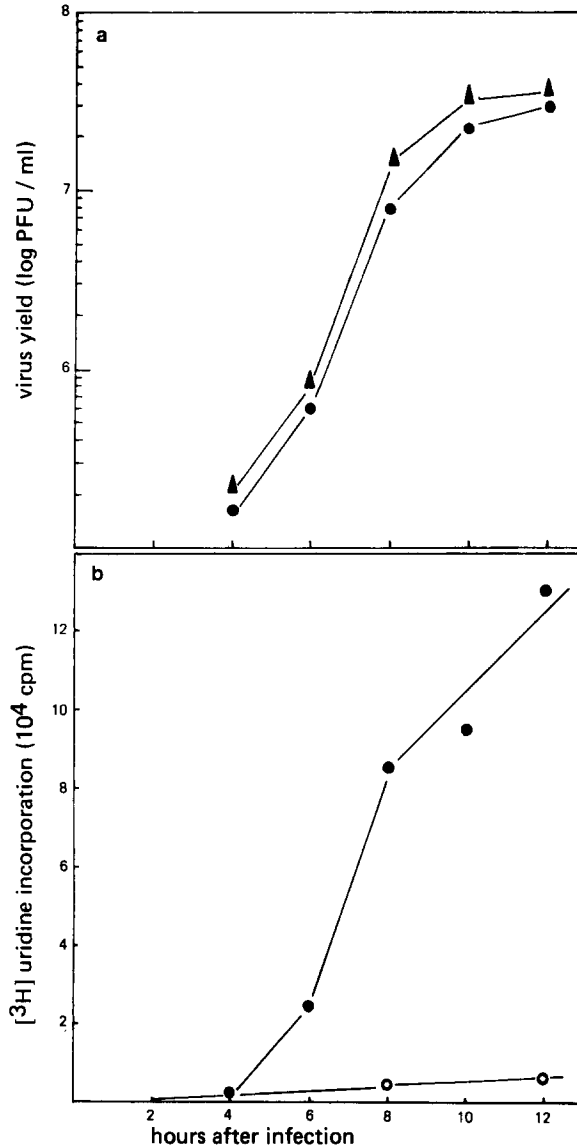


FIG. 1. Growth kinetics of MHV-A59 in *Sac*<sup>-</sup> cells (A). The cells were infected at an m.o.i. of 10 PFU/cell and incubated in the absence of act D (▲) or this drug was added 1 hr pi (●). In the latter case the kinetics of the synthesis of viral RNA were also measured by following the incorporation of [<sup>3</sup>H]uridine added to the medium at 2 hr pi. The results are shown in (B). (○) Mock-infected; (●) infected.

cant difference in final virus yield or in kinetics of virus growth (Fig. 1A).

#### *Kinetics of Virus-Specific RNA Synthesis*

The kinetics of synthesis of intracellular virus-specific RNA in Sac(-) cells were measured in the same experiment. Cells were labeled with [<sup>3</sup>H]uridine in the presence of act D. The synthesis of virus-specific RNA started at 4 hr pi and continued until at least 12 hr pi (Fig. 1B). At this time there was a 21-fold stimulation of the [<sup>3</sup>H]uridine incorporation as compared with the background in mock-infected cells.

#### *Analysis of Intracellular MHV-A59-Specific RNA; Isolation of Poly(A)-Containing RNA Species*

To determine the species of virus-specific RNA formed in Sac(-) cells, in-

fectected act D-treated cells were labeled with [<sup>3</sup>H]uridine between 6 and 8 hr pi. At the end of this period total RNA was extracted from the cells, denatured with glyoxal and Me<sub>2</sub>SO, and analyzed by agarose gel electrophoresis. Several virus-specific RNA species were found in infected but not in mock-infected cells (Fig. 2A, lane 1 and 2). The virus-specific RNA species present in infected cells were further characterized by oligo(dT)-cellulose chromatography. Forty eight percent of the material was found to bind the column, while less than 1% of the RNA obtained from uninfected cells labeled without act D treatment was bound. This material was eluted with low salt buffer and analyzed by electrophoresis in an agarose gel. It contained seven virus-specific RNA species (Fig. 2A, lane 3). It is likely therefore

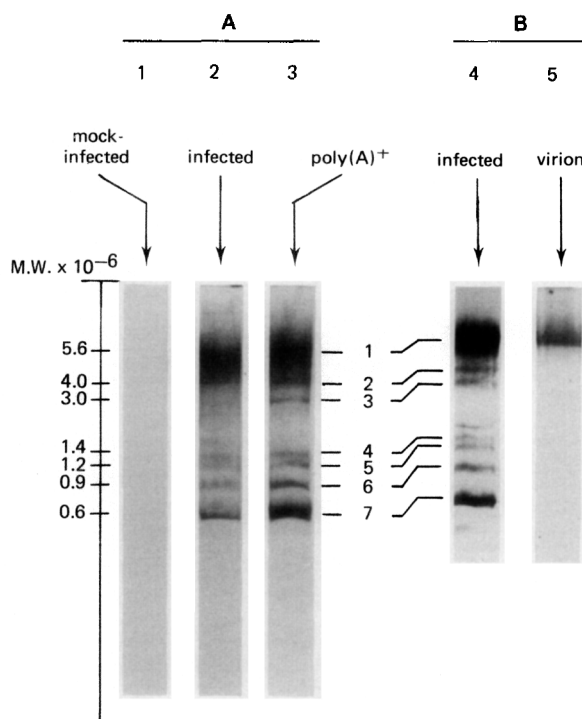


FIG. 2. Electrophoresis in an agarose gel without urea of glyoxal-Me<sub>2</sub>SO denatured MHV-A59 specific intracellular RNA (A). Total RNA, labeled in the presence of actinomycin D, from mock-infected cells was run in lane 1, RNA from infected cells in lane 2. Equivalent amounts of RNA, obtained from about 10<sup>8</sup> cells, were analyzed. RNA extracted from Sindbis virus and rRNAs from Sac(-) cells were used as markers to calculate the molecular weights. Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography from a portion of the material run in lane 2. This material was run in lane 3. In (B) RNA isolated from purified virus and virus-specific RNA from cells were compared by electrophoresis in the same urea-containing gel. The largest intracellular virus-specific RNA comigrated with virion RNA.

that the seven virus-coded intracellular RNAs are mRNAs. The apparent molecular weights of these RNAs were determined using cellular 18 S and 28 S rRNA and 42 S RNA from Sindbis virus as markers. These markers have molecular weights of  $0.67 \times 10^6$ ,  $1.75 \times 10^6$  (McMaster and Carmichael, 1977), and  $4.3 \times 10^6$  (Kääriäinen and Söderlund, 1978), respectively. The molecular weights of the seven viral RNAs were in millions: 5.6 (RNA1), 4.0 (RNA2), 3.0 (RNA3), 1.4 (RNA4), 1.2 (RNA5), 0.9 (RNA6), and 0.6 (RNA7). Figure 2A shows that purification of the RNAs on oligo(dT)-cellulose leads to sharper bands except for RNA1. This RNA remains present as a broad and diffuse band. This could be due to the presence of A-U base pairs which are not denatured with glyoxal. Therefore we have used agarose gels containing 6 M urea for electrophoresis. From comparing lane 2 and 4 from Fig. 2 it is clear that the presence of urea indeed results in sharper bands also for RNA1. Therefore, we have used urea-containing gels in all subsequent experiments. Using

this system we have compared the intracellular virus-specific RNAs to virion RNA. The largest RNA comigrated with the viral genome (Fig. 2B).

There were two kinds of artifacts that we wanted to exclude, namely (a) that defective interfering (DI) particles had accumulated in our virus stocks and (b) that the six smaller viral RNA species were degradation products of the genome sized  $5.6 \times 10^6$  dalton RNA. Possibility (a) could be ruled out since RNA extracted from virions grown in a replicate culture of cells that made the seven virus-specific RNAs contained only one species of RNA, with a mass of  $5.6 \times 10^6$  daltons (Fig. 2B); if DI particles had been formed smaller RNAs would have been found. The following control experiments to test (b) were carried out: Sac(-) cells were infected with MHV-A59 as described above; act D was added but no [ $^3$ H]uridine. Just before lysis of the cells [ $^3$ H]uridine-labeled MHV-A59 was added and RNA was extracted from the mixture and analyzed. Virion RNA was not degraded in the extract of infected cells.

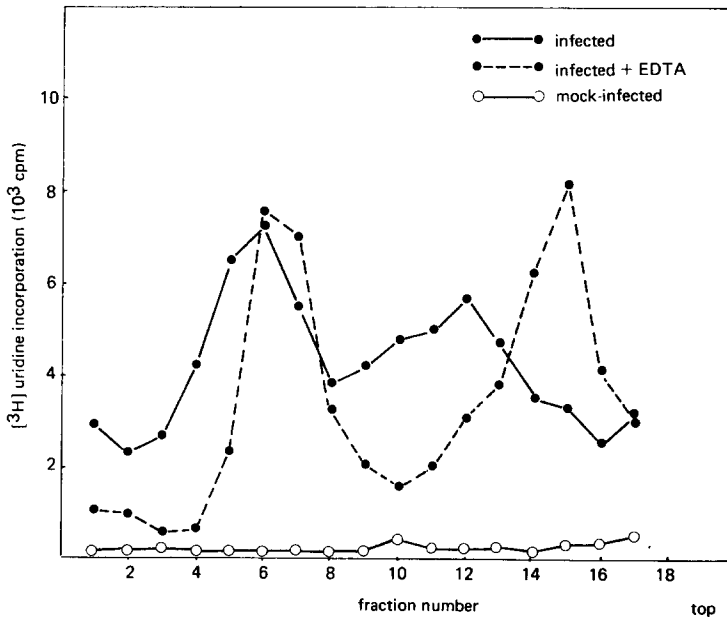


FIG. 3. Sucrose gradient analysis of polysomes containing viral mRNA. Cytoplasmic fractions from cells treated with act D and labeled with [ $^3$ H]uridine were compared. (○—○) Mock-infected; (●—●) infected. EDTA was added to a lysate of infected cells and this material was also run (●---●).



In another experiment Sac(-) cells were infected with Sindbis virus and the cells were labeled between 6 and 8 hr pi in the presence of act D. RNA was extracted and analyzed in the same way as described for MHV-A59 intracellular RNA. Only the well known 42 S and 26 S RNA species (Kääriäinen and Söderlund, 1978) were found.

To determine if double-stranded RNA was present in the postnuclear supernatant of infected cells, the RNA was digested with pancreatic ribonuclease A (1  $\mu\text{g}/\text{ml}$  at 37° for 30 min) and then electrophoresed in an agarose gel without denaturation. No RNA was detected after this treatment.

#### *Isolation of Virus-Specific Polyribosomes and Analysis of mRNAs*

To obtain more direct evidence for a messenger function of the seven RNAs, we have looked at virus-specific mRNA present in polyribosomes. The postnuclear supernatants from control cells and virus-infected act D-treated cells labeled with [<sup>3</sup>H]uridine were analyzed by sedimentation in iso-

kinetic sucrose gradients (Fig. 3). As expected, no [<sup>3</sup>H]uridine was incorporated into RNA from mock-infected cells. In gradients loaded with lysates from infected cells two peaks were present: one sedimenting from 80 to 200 S and one at about 230 S. To determine whether the RNA sedimenting in the gradient was ribosome-associated, EDTA was added to the cell extract before analysis. The slower sedimenting material was EDTA-sensitive and remained at the top of the gradient. Treatment with EDTA did not alter the sedimentation of the bulk of the material present in the 230 S peak, indicating that this RNA is not polyribosome-associated (Fig. 3). RNA extracted from every fourth gradient fraction was analyzed by electrophoresis in an agarose gel (Fig. 4A). Seven virus-specific RNA species were detected throughout the sucrose gradient; they had the same apparent molecular weights as the intracellular RNAs described above.

The mRNA species released with EDTA and the RNA present in the EDTA-resistant peak were also analyzed. The

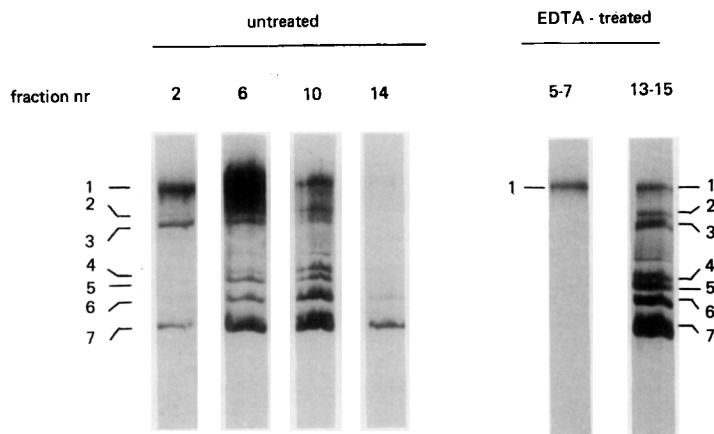


FIG. 4. Agarose gel electrophoresis of glyoxal-Me<sub>2</sub>SO denatured RNA extracted from polyribosome gradients. Virus-specific mRNAs were labeled and polyribosomes from MHV-A59 infected cells were analyzed as shown in Fig. 3. RNA was extracted, denatured, and analyzed by agarose gel electrophoresis. The left-hand part of the figure gives the results for every fourth fraction of the gradient shown in Fig. 3. The numbers 1 to 7 indicate the position of the seven virus-specific poly(A)-containing RNAs isolated from infected cells (Fig. 2) which were run as markers in the same gel. The right-hand portion represents an analysis of the RNAs present in the two peaks found after the addition of EDTA to the cell lysate. The fraction numbers refer to the gradients shown in Fig. 3. Equal amounts of radioactivity were loaded on the gel but the lane containing the EDTA-resistant material was only exposed for 12 hr, compared to 96 hr for the RNAs present in the EDTA-sensitive peak.

EDTA-resistant material contained only one RNA species of  $5.6 \times 10^6$  daltons (Fig. 4B). It is likely therefore that this material consists of subviral ribonucleoprotein. A small portion of this RNA (9% determined by microdensitometry of a fluorograph) together with all of the six smaller RNAs were found at the top of the gradient after EDTA treatment. It can be concluded that all RNA species are indeed mRNAs.

#### DISCUSSION

The most surprising finding of this study is that MHV-A59 synthesizes six subgenomic mRNA species. We were able to show that these six poly(A)-containing virus-specific RNAs are no artifacts caused by defective interfering particles. Degradation of the genome-sized RNA to the smaller RNAs does not occur, since virion RNA added to a lysate of infected cells is extracted undegraded. With our extraction procedure we could also extract the genomic RNA and the subgenomic mRNA from Sindbis virus-infected Sac(-) cells. Finally the uv target size of the RNAs decreases with their molecular weight (C. E. Jacobs, W. J. M. Spaan, and B. A. M. van der Zeijst, unpublished data). This also rules out degradation of the largest RNA, although the smaller RNAs could arise from processing of the  $5.6 \times 10^6$  daltons RNA. Genomic RNA of MHV is infectious and therefore it must function as a messenger (Wege *et al.*, 1978). However, only a small fraction of the genome-sized RNA

synthesized between 6 and 8 hr pi is found in polysomes.

Denaturation of RNA with glyoxal- $\text{Me}_2\text{SO}$  excludes a number of artifacts in electrophoresis and results in accurate molecular weights (McMaster and Carmichael, 1977). We found that electrophoresis of glyoxal denaturated RNA in urea-containing gels is a even superior to the original method. The molecular weight found for the genome— $5.6 \times 10^6$ —agrees well with the value of  $5.4 \times 10^6$  found by Lai and Stohlman (1978), also using denaturing conditions, and the value of  $5.4 \times 10^6$  to  $6.5 \times 10^6$  determined for MHV-4 under nondenaturing conditions by Wege *et al.* (1978).

Addition of the values obtained by this method for the six subgenomic mRNAs results in a value of  $11.1 \times 10^6$ , which is almost twice the molecular weight of the genome. This implies that there are sequence homologies between the subgenomic mRNAs. We have no data so far to indicate where these homologies are located.

Even while these data are still lacking one may speculate about the replication strategy of coronaviruses. Figure 5 represents our working hypothesis. The two essential points of the model are:

(1) The genome and all mRNAs share a homologous region of  $0.6 \times 10^6$  daltons at their 3'-termini.

(2) Internal initiation sites for protein synthesis are not accessible; in each mRNA only the 5'-terminal region not present in the next smaller RNA is translated.

Our model partly is based on an analogy

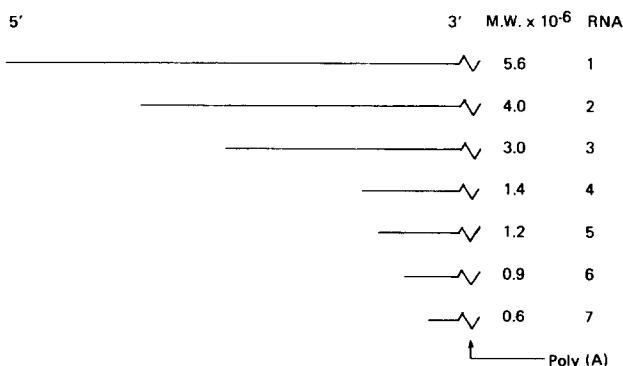


FIG. 5. A model for the sequence homology between mouse hepatitis virus mRNAs.

with the replication mechanism of togaviruses (Kääriäinen and Söderlund, 1978) and tobacco mosaic virus (Hunter *et al.*, 1976). These viruses have one subgenomic mRNA representing the 3'-terminal portion of the genome. Under normal conditions the internal initiation site on the genome remains cryptic. There are also certain analogies with the subgenomic *env*-messenger in retroviruses (Rothenberg *et al.*, 1978) and the mRNA for VP3 of polyoma and SV40; this mRNA represents the 3'-terminal part of the messenger for VP2 (Fiers *et al.*, 1978; Deininger *et al.*, 1979).

Experimental corroboration comes from Siddell *et al.* (1980) who have presented evidence for the involvement of two, and probably more, subgenomic mRNAs in the replication of MHV-4. These two mRNAs are monocistronic; a 17 S RNA encoding the 60K nucleocapsid protein and a 19 S RNA encoding a 23K structural protein were found. They are probably equivalents of our RNA7 and RNA6, respectively. We have separated all seven viral mRNAs and injected each RNA in oocytes of *Xenopus laevis* (P. J. M. Rottier, W. J. M. Spaan, M. C. Horzinek, and B. A. M. van der Zeijst, manuscript in preparation). In this way we were able to demonstrate directly that RNA3, RNA6, and RNA7 are functional mRNAs. In agreement with our model RNA3 coded for a 150,000 dalton protein, RNA6 for a 23–26,000 dalton protein and RNA7 for a 55,000 molecular weight protein. We are currently characterizing these products. Experiments are also in progress to establish the location of the homologous RNA sequences in the genome and in the subgenomic mRNAs.

#### NOTE

Stern and Kennedy (*J. Virol.* **34**, 665–674, 1980) have shown that cells infected with the avian coronavirus infectious bronchitis virus contain six major virus-specified RNAs, presumably viral mRNAs. The molecular weights of these RNAs roughly correspond with those found by us for the MHV-A59 mRNAs with the exception that RNA2 seems to be lacking in the

avian system. These authors also provide evidence that the sequence of each RNA is contained within the sequences of all larger RNA species and refer to preliminary observations that the subgenomic RNAs share the 3' end of the genome. These data support the model we have presented.

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