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The Virus-Specific Intracellular RNA Species of Two Murine Coronaviruses: MHV-A59 and MHV-JHM[1]

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Seven virus-specific, polyadenylated RNA species have been identified in mouse cells infected with the murine coronaviruses MHV-A59 (A59V) or MHV-JHM (JHMV). MHV-infected 17CL-1 cells were labeled with [^{32}P]orthophosphate in the presence of actinomycin D and the cytoplasmic RNA was extracted and analyzed by agarose gel electrophoresis. These RNA species range in size from 6.3×10^5 to 6.1×10^6 daltons. The A59V and JHMV-specific RNAs have identical molecular weights and comigrate in agarose gels. The largest intracellular RNA species is identical to RNA isolated from purified virions, as determined by agarose gel electrophoresis and oligonucleotide fingerprint studies of ribonuclease T_1 digests. Oligonucleotide fingerprints of the six subgenomic RNAs show that the sequences they contain are present in virion RNA, confirming their virus-specific nature. The fingerprinting studies also demonstrate that the six subgenomic RNA species make up a nested set. The sequences present in each RNA species are also present in all larger RNA species. These larger RNAs also contain additional sequences consistent with their greater size. The subgenomic RNAs fulfull many of the criteria for mRNAs. Possible mechanisms for generating these RNAs are discussed.

INTRODUCTION

Coronaviruses are widespread in nature and have been associated with several diseases in infected hosts (Robb and Bond, 1979a). Coronaviruses are defined as pleomorphic enveloped particles about 100 nm in diameter which have characteristic bulbous projections on their surface, bud through the endoplasmic reticulum rather than the plasma membrane, and contain RNA (Tyrell *et al.*, 1978). Several studies of coronavirus genomic RNA have been reported (Lomneczi, 1977; Lomneczi and Kennedy, 1977; Yogo *et al.*, 1977; Lai and Stohlman, 1978; Macnaughton and Madge, 1978; Schochetman *et al.*, 1977; Tannock and Hierholzer, 1978; Guy and Brian, 1979; Wege *et al.*, 1978). These studies have indicated that the coronavirus genome is a large ($5.4\text{-}8.1 \times 10^6$ daltons) single-stranded RNA which is polyadenylated and is infectious. These properties identify the coronaviruses as positive-stranded RNA viruses.

In contrast to the data obtained on coronavirus virion RNA, few data have been published on intracellular coronavirus-specific RNA. Mishra and Ryan (1973) reported that porcine kidney cells infected with transmissible gastroenteritis virus (TGEV) contained actinomycin D-resis-

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tent RNA species which sedimented between 18 and 28 S. Robb and Bond (1979b) have studied murine hepatitis virus (MHV)-infected cells and found that deproteinized RNA from virus-specific polysomes sedimented between 10 and 28 S. RNA in this size range was recently demonstrated to code for two MHV structural proteins (Siddell *et al.*, 1980). Stern and Kennedy (1980a,b) have identified six virus-specific RNA species in cells infected with avian infectious bronchitis virus (IBV).

We have been studying the virus-specific RNA species synthesized in cells infected with two strains of MHV:MHV-A59 (A59V) and MHV-JHM (JHMV). JHMV is highly neurotropic and produces an encephalomyelitis with demyelination in its natural host, the mouse (Bailey *et al.*, 1949). A59V is weakly neurotropic (Robb *et al.*, 1979). We have been investigating the replication of these viruses in the hope of eventually understanding the molecular mechanisms by which JHMV produces demyelination.

We report here the identification of seven major MHV-specific intracellular RNA species. These RNAs have been characterized by agarose gel electrophoresis, poly(U) Sepharose affinity chromatography, and oligonucleotide fingerprinting.

MATERIALS AND METHODS

Materials. [^{3}H]Uridine was obtained from New England Nuclear and [^{32}P]-orthophosphate (carrier free) from New England Nuclear, Amersham, or ICN. Phenol was obtained from Mallinckrodt and redistilled prior to use. Actinomycin D and ribonuclease T_1 (Sankyo) were purchased from Calbiochem and hexadecyltrimethylammonium bromide (CTAB) and poly(U) Sepharose from Sigma. Glyoxal was purchased from Fisher Chemicals as a 40% solution and deionized by the method of McMaster and Carmichael (1977) before use. Methyl mercuric hydroxide was purchased from Alpha Chemicals, low melting point agarose from Bethesda Bioresearch Laboratories, standard low M_r agarose from Bio-Rad, linear polyacrylamide from British Drug House, and proteinase K from Beckman Chemicals. Acrylamide, xylene cyanol FF, and bromphenol blue were obtained from Eastman Chemical. Hydroxylapatite (HA; DNA grade) was obtained from Bio-Rad and prepared as described by Stern and Kennedy (1980a).

Cells. The origin and growth of the murine cell line 17CL·1 has been previously described (Sturman and Takemoto, 1972; Bond *et al.*, 1979). HeLa cells were obtained from Dr. John Holland and grown in 32-oz. prescription bottles in Dulbecco's modified Eagle's medium with 10% calf serum.

Virus. The origin and growth of A59V and JHMV virus stocks have been described (Robb and Bond, 1979b). For experiments 17CL·1 cells were removed from the glass substrate with 0.1% twice crystallized trypsin in Puck's saline A supplemented with 0.01 *M* Tris (pH 8.1) and 0.1% EGTA [ethyleneglycol-bis(β-amino ethyl ether)-*N,N'*tetraacetic acid], resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DME10), centrifuged at 500 *g* for 2 min and resuspended in medium containing 2% fetal bovine serum (DME2). Sufficient virus was added to give a multiplicity of infection (m.o.i.) between 0.1 and 0.15 plaque forming units/cell. Virus adsorption was at 37° for 30 min. Following adsorption the cells were centrifuged at 500 *g* for 2 min, resuspended in DME prewarmed to 37°, and plated into 35-mm (1.5×10^6 cells/dish), 60-mm (5×10^6 cells/dish), 100-mm (1.5×10^7 cells/dish), or 150-mm ($4\text{–}5 \times 10^7$ cells/dish) plastic culture dishes and further incubated at 37°. For most experiments utilizing [^{32}P]orthophosphate as label the cells were resuspended and plated in phosphate-free DME-2.

The strain of mengovirus used was that described by Plagemann and Swim (1966). Virus stocks were grown in 17CL·1 cells infected at an m.o.i. of 10^{-4} PFU/cell.

Vesicular stomatitis virus (VSV), Indiana serotype, was kindly supplied by Dr. John Holland. The virus was grown in BHK-21 cells infected at an m.o.i. of 10^{-4} PFU/cell.

Adenovirus type 2 (Ad2) was obtained from Dr. Marshall Horwitz and grown in HeLa cell monolayers infected at an m.o.i. of 1–5 PFU/cell.

Extraction of intracellular RNA. Cytoplasmic extracts of infected or mock-infected 17CL·1 cells were prepared using NP-40 (Borun *et al.*, 1967). Monolayers were rinsed once with cold phosphate-buffered saline (PBS), scraped into cold reticulocyte standard buffer (RSB, 0.01 *M* Tris, pH 7.4, 0.01 *M* NaCl, 0.0015 *M* $MgCl_2$) with a rubber policeman and transferred to a tube containing sufficient NP-40 to give a final concentration of 1%. The cell suspension was vortexed gently, incubated on ice for 5 min, vortexed again, and the nuclei removed by centrifugation at 1500 *g* for 2 min. The cytoplasmic extract was adjusted to 1% SDS, 0.4 *M* NaCl, 0.01 *M* EDTA, and 1.0 mg/ml proteinase K and incubated at 50° for 5 min and at room temperature for an additional 25 min. The RNA was then extracted with phenol chloroform (Robb and Bond, 1979b) and precipitated with 3 volumes of ethanol at −20° in the presence of 50 μg of yeast carrier tRNA.

Preparation of MHV virion RNA. 17CL·1 cells were infected with A59V or JHMV in phosphate-free DME2 as described above. Following virus adsorption the cells were plated in 100- or 150-mm culture dishes and incubated at 33°. At 4 hr post-infection (hpi) [^{32}P]orthophosphate was added to a concentration of 100–1000 μCi/ml. At 16–18 hpi the cell-associated virus was released by two cycles of freeze thawing and the resulting lysate clarified by centrifugation at 10,000 *g* for 30 min at 4°. Virus was concentrated by centrifugation for 60 min at 35,000 rpm in the SW40 rotor through a 0.5-ml pad of 15% (w/w) potassium tartrate in MSE buffer (0.01 *M* morpholinopropane sulfonic acid, 0.15 *M* NaCl, 0.001 *M* EDTA, pH 6.8). The virus pellets were resuspended by sonication in 0.5 ml of MSE buffer, layered onto a 12 ml gradient of 5–25% (w/w) potassium tartrate in MSE buffer, and centrifuged at 35,000 rpm for 45 min in the SW40 rotor. The gradient was fractionated and the virion peak located by counting aliquots of each fraction. For the preparation of highly purified virus this material was diluted with MSE, layered on a 9 ml 10–40% (w/w) potassium tartrate gradient, and centrifuged in the SW40 rotor at 37,000 rpm for 4 hr. The gradient was fractionated, aliquots were counted, and the peak of radiolabeled virus collected. This material had a buoyant density between 1.19 and 1.17 g/cm^3. The virus was diluted with MSE buffer, pelleted at 45,000 rpm for 30 min in the SW50.1 rotor, and resuspended by sonication in 1 ml of MSE buffer. Virion RNA was extracted in a similar manner to intracellular RNA and precipitated with ethanol after the addition of 50 μg of carrier tRNA.

Virion RNA for fingerprinting was prepared by a slightly different procedure. Virus was concentrated and banded on a 5–25% potassium tartrate gradient and this partially purified virus was diluted with MSE buffer, pelleted in the SW50.1 rotor, and the RNA was extracted and precipitated with ethanol as described above. The RNA was collected by centrifugation, dried under a stream of nitrogen, and dissolved in 0.1 ml of SDS buffer (0.01 *M* Tris, 0.01 *M* NaCl, 0.001 *M* EDTA, 0.1% SDS, pH 7.4). It was then overlaid on a 5-ml 10–30% (w/w) sucrose in SDS buffer gradient and centrifuged at 46,000 rpm for 107 min at 20° in the SW50.1 rotor. The gradient was fractionated and the peak of 48 S virion RNA (Robb and Bond, 1979b) located by counting aliquots of each fraction. These fractions were pooled, adjusted to 0.4 *M* NaCl and precipitated with ethanol in the presence of 100 μg of tRNA carrier. This material was used for fingerprinting studies and was homogeneous upon analytical electrophoresis.

Isolation of poly(A)-containing RNA. Intracellular RNA was extracted and precipitated with ethanol as described. The polyadenylated RNA species were isolated by affinity chromatography over poly(U) Sepharose as described by Wilt (1977).

Agarose Gel Electrophoresis

1. Analytical electrophoresis. Agarose gel electrophoresis following glyoxal denaturation was essentially as previously described (McMaster and Carmichael,

1977). Nucleic acids were recovered from ethanol by centrifugation, dried under a stream of nitrogen and dissolved in glyoxal buffer. RNA or DNA was reacted with glyoxal at 50° for 5 min followed by an additional 55 min at room temperature. Samples were electrophoresed at 100 V for 4 hr in horizontal slab gels containing 0.7, 0.8, or 1.0% agarose, 0.01 *M* phosphate buffer, pH 7.0, 0.002 *M* EDTA. When appropriate, the gels were strained with acridine orange and the positions of rRNA were marked with stainless-steel wire clips inserted into the gel. The gels were fixed in ethanol, dried under vacuum, and exposed to Kodak XR film at room temperature.

To quantitate the individual RNA species present in cytoplasmic extracts two methods were used. Autoradiographs were prepared with two exposure times to ensure linearity of the film and scanned with a densitometer (E. C. Corporation). The area under the peaks was quantitated by cutting out and weighing tracings of the scans. Alternatively, the regions of the gel corresponding to the bands present in the autoradiographs were excised and the amount of RNA present in the band was quantitated by liquid scintillation counting. These methods gave similar results. Molar ratios of RNA species were calculated by dividing the counts per minute or weight of paper in each peak by its molecular weights $\times 10^{-6}$ and normalizing all values such that the major RNA species, RNA7 (see Results), was set at 100.

2. Preparative electrophoresis. Labeled intracellular RNA was extracted from 1.2 $\times 10^8$–1.6 $\times 10^8$ cells, the poly(A)-containing RNA species selected by affinity chromatography, and then electrophoresed in gels containing 1% low melting point agarose. Two gel systems were used. In initial experiments the phosphate buffer system used for analytical agarose electrophoresis was employed without prior denaturation of the RNA with glyoxal and dimethyl sufoxide (McMaster and Carmichael, 1977). Electrophoresis was for 4 hr at 100 V. The above conditions did not reproducibly allow complete resolution of all virus-specific RNA species. Later experiments employed methyl mercury denaturation of the RNA and electrophoresis in agarose gels containing 5 m*M* methyl mercuric hydroxide (Bailey and Davidson, 1976). Electrophoresis was at 100 V for 6 hr in a horizontal 1% low melting point agarose gel. Following electrophoresis, the majority of the methyl mercury was removed from the gel by soaking in two changes of 750 ml sterile 0.5 *M* ammonium acetate (Bailey and Davidson, 1976).

For both gel systems RNA species were located by autoradiography of the wet gels wrapped in Saran. Agarose strips corresponding to the bands seen in the autoradiographs were cut from the gel with a flamed scalpel and placed in tightly capped screw top tubes. RNA was recovered from gels by melting the agarose at 70° for 5 min. Five milliliters of a slurry of HA, prewarmed to 37°, was added to each tube. Samples containing methyl mercury had 2-mercaptoethanol added to a concentration of 1%. The mixture was cooled to 37°, HA was collected by centrifugation, and washed twice with Tris acetate buffer (10 m*M* Tris, 20 m*M* sodium acetate, 5 m*M* EDTA, pH 7.3) in a 37° warm room to remove the liquified agarose. For samples which had been treated with methyl mercury, the Tris acetate buffer was supplemented with 1% 2-mercaptoethanol. The HA was resuspended in 5 ml of Tris acetate buffer and transferred to ethanol-washed disposable columns (Quick-sep, QS-Q obtained from Isolab) placed in heat sterilized 16 × 125-mm culture tubes. The culture tubes containing the columns were centrifuged at 1000 *g* for 10 min at room temperature to remove the Tris acetate buffer. The RNA was eluted from HA columns by two washes of 1.0 ml of 0.4 *M* sodium phosphate, 1 m*M* EDTA (pH 7.0) which were similarly forced from the columns by centrifugation at 1000 *g* for 10 min. The phosphate eluates were pooled and the RNA precipitated with CTAB as described by Stern and Kennedy (1980a). The CTAB precipitates were dissolved in 0.2 ml of 50 m*M* Tris, pH 7.4, containing 1 *M* NaCl and 1 m*M* EDTA and the RNA precipitated with 1.0 ml of ethanol.

Ribonuclease T_1 fingerprinting. RNA was recovered from ethanol by centrifugation, dissolved in 0.1 ml of sterile water,

transferred to a sterile 1.5-ml Eppendorf centrifuge tube, and the water was evaporated with a stream of nitrogen. The RNA was digested with 10 μl of ribonuclease T_1 (1 mg/ml) in 10 m*M* Tris, pH 7.6, at 37° for 30 min. Ten microliters of a solution containing 5 *M* urea, 50% sucrose, 0.1% bromphenol blue, and 0.1% xylene cyanol FF was added to the digestion products. The RNase T_1-resistent oligonucleoties were separated by two-dimensional polyacrylamide gel electrophoresis as described by Stern and Kennedy (1980a) with the following modification. After electrophoresis in the first dimension the gel strips were washed for 25 min with two changes of 100 m*M* Tris borate buffer containing 2.5 m*M* EDTA, pH 8.3, prior to pouring the second dimension gel (Lee *et al.*, 1979).

Following electrophoresis the gels were wrapped with polyethylene sheets and exposed at −70° to Kodak XR film in the presence of Cronex lightning plus (DuPont) intensifying screens (Laskey and Mills, 1977). Exposures ranged from 2 days to 3 weeks.

Preparation of molecular weight markers. Mengovirus was purified from infected 17CL·1 cells labeled with [^{32}P]orthophosphate from 4 to 16 hpi. The culture fluids were clarified by centrifugation at 10,000 *g* for 30 min. Sodium dodecyl sulfate was added to the clarified fluids to a concentration of 0.1% and the virus pelleted through a 1.5-ml 30% sucrose pad by centrifugation at 25,000 rpm in the SW 27.1 rotor for 3 hr at 20°. The virus was resuspended in 1 ml of 0.01 *M* Tris buffer, pH 7.4, layered onto a 16-ml cesium chloride gradient (1.2–1.4 g/cm^3) and centrifuged overnight in the SW27.1 rotor at 24,000 rpm. The gradient was fractionated, aliquots were counted and the peak of radioactive virus at a density of 1.34 g/cm^3 was pooled. This material was diluted with 0.01 *M* Tris, pH 7.4, and pelleted in the SW50.1 rotor at 45,000 rpm for 1 hr. The pelleted virus was resuspended in 0.01 *M* Tris buffer, the RNA deproteinized with SDS and proteinase K, extracted with phenol chloroform, and precipitated with ethanol. The RNA was collected by centrifugation and prepared for analytical electrophoresis.

VSV RNA was prepared from VSV-infected BHK 21 cells labeled with [^{32}P]orthophosphate from 2 to 16 hpi. Culture fluids were clarified by centrifugation at 10,000 *g* for 30 min. VSV was pelleted from the clarified supernate by centrifuging in the SW40 rotor at 37,000 rpm for 1 hr, the virus pellet was taken up in 0.01 *M* Tris buffer, pH 7.4, the RNA deproteinized with SDS and proteinase K, and precipitated with ethanol. This VSV RNA preparation was used as a molecular weight marker without further purification although it was contaminated with significant amounts of 28 S and 18 S RNA.

HeLa cells were infected with Ad2 and labeled with [^{32}P]orthophosphate from 16 to 40 hpi. Infected cells were scraped from the substrate, pelleted by centrifugation, and resuspended in 1 ml of 0.01 *M* Tris, pH 8.1. The cells were disrupted by sonication and the labeled virus was purified as previously described (Horwitz, 1971). The purified virus was diluted with 0.01 *M* Tris, pH 8.1, and pelleted by centrifugation at 37,000 rpm for 1 hr. The virus was resuspended in 1 ml of 0.01 *M* Tris buffer (pH 7.4), extracted with phenol (Petterson and Sambrook, 1973), and the DNA precipitated with ethanol. The DNA was recovered from ethanol by centrifugation and denatured with glyoxal prior to electrophoresis.

Ribosomal RNA was purified from cytoplasmic extracts of 17CL·1 or BHK-21 cells which were labeled for 24 hr with [^{32}P]orthophosphate. SDS was added to cytoplasmic extracts to a final concentration of 1% and the 18 and 28 S rRNA were purified by centrifugation through 5–30% (w/w) sucrose gradients (containing 0.01 *M* Tris, 0.01 *M* NaCl, 0.001 *M* EDTA, 0.1% SDS, pH 7.4) at 37,000 rpm for 4 hr in the SW40 rotor at 20°. The 28 and 18 S peaks were located by counting aliquots of the gradients, treated with proteinase K and extracted with phenol chloroform. The RNA was precipitated with ethanol, collected by centrifugation, and denatured with glyoxal prior to electrophoresis.

Escherichia coli rRNA was purchased from Miles Biochemicals and denatured

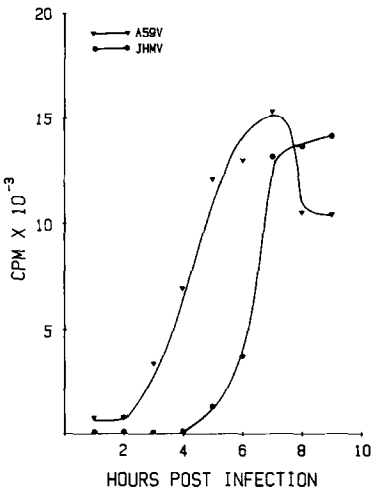

FIG. 1. Kinetics of actinomycin D-resistant [^{3}H]uridine incorporation. 17CL·1 cells were infected with A59V, JHMV, or mock-infected, resuspended in DME2, seeded in replicate 35-mm dishes, and incubated at 37°. At 1 hr intervals postinfection actinomycin D was added to 5 μg/ml to triplicate dishes. The dishes were incubated for 15 min and then labeled for 1 hr with 5 μCi/ml of [^{3}H]uridine. At the end of the labeling period the dishes were removed from the incubator, washed twice with cold phosphate-buffered saline (PBS), scraped from the dishes into cold PBS, and frozen at −20°. The samples were thawed 24 hr later and the amount of trichloroacetic acid precipitable radioactivity determined. The results are expressed as the mean of triplicate samples. The amount of radioactivity incorporated into mock infected dishes (about 2000 cpm) has been substracted from the values shown.

with glyoxal prior to electrophoresis. The 16 and 23 S rRNAs were located following electrophoresis by staining with acridine orange and their locations marked with wire clips inserted in the gel.

RESULTS

Kinetics of Actinomycin D-Resistant [^{3}H]-Uridine Incorporation

Robb and Bond (1979b) have shown that MHV-specific RNA synthesis is resistant to actinomycin D. We have taken advantage of this to determine the kinetics of MHV-specific RNA synthesis by assaying the incorporation of [^{3}H]uridine (in the presence of actinomycin D) into acid-precipitable material. 17CL·1 cells were infected with JHMV, A59V, or mock infected and at hourly intervals replicate dishes were incubated for 15 min with actinomycin D and then labeled for 1 hr in the presence of the drug. The results of this experiment are shown in Fig. 1. After an initial lag phase of 3 hr in A59V-infected cells and 5 hr in JHMV-infected cells, actinomycin D-resistant uridine incorporation increases exponentially until 7 hpi, when it plateaus. These results are consistent with the one step growth curves of these two viruses (Bond *et al.*, 1979). In view of the above results, we routinely labeled cells from 4 to 8 hpi in subsequent experiments.

Identification of MHV-Specific RNA Species

A59V, JHMV, and mock-infected cells were labeled with [^{32}P]orthophosphate from 4 to 8 hpi in the presence of actinomycin D. The cytoplasmic RNA was extracted, denatured with glyoxal, and analyzed by electrophoresis on an agarose gel (Fig. 2). Seven RNA species, designated 1-7 in decreasing order of size, were reproducibly present in JHMV- and A59V-infected cells. JHMV-infected cells synthesize less RNA5 than A59V-infected cells (Table 1). Although the band representing JHMV-specific RNA5 is barely visible in Fig. 2 (lane J), this RNA species could be seen with a longer exposure of the autoradiograph, was reproducibly present in infected cell extracts, and can be better seen in Fig. 6. In addition, three minor RNA species were often observed, although not in every experiment. The largest of these RNAs migrated between RNAs 3 and 4 just faster than 28 S rRNA. Two minor RNAs migrating faster than RNA7 were also sometimes observed. These RNAs were not studied extensively due to difficulty in preparing sufficient material and their inconsistent appearance. No discrete bands were identified in mock-infected extracts. The largest MHV-specific RNA, RNA 1, coelectrophoreses with virion RNA and represents the intracellular form of the genome. In contrast to the size differences we have observed between A59V-

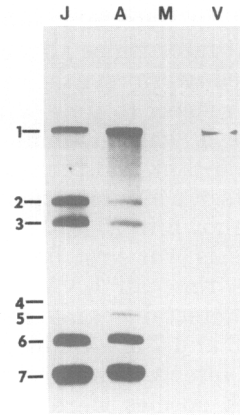

FIG. 2. Electrophoretic analysis of A59V- and JHMV-specific intracellular RNAs. Sixty-millimeter dishes of A59V, JHMV, or mock-infected 17CL·1 cells were labeled from 4 to 8 hr postinfection with 300 μCi of [^{32}P]orthophosphate in the presence of 5 μg/ml actinomycin D. The cytoplasmic RNA was extracted, precipitated with ethanol, denatured with glyoxal, and analyzed by electrophoresis through a 0.8% agarose gel as described under Materials and Methods. Lane V represents RNA extracted from purified JHMV virions. Lanes A, J, and M represent the RNA from A59V and JHMV and mock-infected cells, respectively. The positions and designations of the MHV-specific RNAs are indicated by the numbers on the right-hand side of the figure.

and JHMV-specific proteins (Bond *et al.*, 1979), A59V- and JHMV-specific RNAs comigrate in agarose gels. The amount of virus-specific RNA synthesized in A59V-infected cells is, however, about 30% greater than that synthesized in JHMV-infected cells as determined by the incorporation of [^{3}H]uridine or [^{32}P]orthophosphate into acid-insoluble cytoplasmic material in the presence of actinomycin D.

The Molecular Weights of MHV-Specific Intracellular RNA

The molecular weights of MHV-specific RNA species 1–7 were determined by agarose gel electrophoresis with appropriate molecular weight markers. Glyoxal denaturation allows the use of DNA as well as RNA size markers (McMaster and Carmichael, 1977). A plot of the $\log_{10}$ molecular weight against electrophoretic mobility is shown in Fig. 3. A linear relationship was obtained over a molecular weight range of 5.5×10^5–1.15×10^7. The molecular weights obtained for the seven major MHV specific RNAs are shown in Table 1.

Cytoplasmic RNA was extracted from infected cells labeled from 4 to 8 hpi and analyzed by agarose gel electrophoresis. The relative molar amounts of the individual MHV-specific RNAs were quantitated as described under Materials and Methods. The results are shown in Table 1. A59V- and JHMV-infected cells synthesize virus-specific RNA species in similar

TABLE 1

THE SIZE AND RELATIVE MOLAR AMOUNTS OF MHV-SPECIFIC RNA

RNA species	Molecular weight	Relative molar amounts[a]	
		A59V	JHMV
Virion RNA	6.1×10^6	—	—
RNA 1	6.1×10^6	3.33 (1.48-4.20)	1.50 (0.66-2.26)
RNA 2	3.4×10^6	2.00 (1.31-2.81)	2.92 (2.63-3.24)
RNA 3	2.6×10^6	3.24 (2.83-5.16)	5.23 (3.32-6.92)
RNA 4	1.2×10^6	3.09 (1.0-4.52)	1.69 (1.14-2.21)
RNA 5	1.08×10^6	11.0 (7.7-22.5)	0.86 (0.23-1.80)
RNA 6	8.4×10^5	32.6 (26.5-38.1)	31.5 (24.7-46.3)
RNA 7	6.3×10^5	100	100

[a] The data presented are the mean of five determinations of preparations labeled from 4 to 8 hr postinfection. The range of values observed is in parentheses.

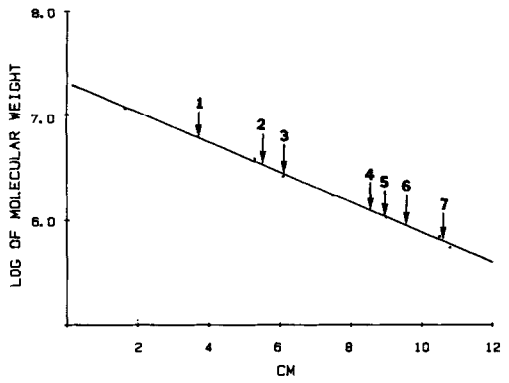

FIG. 3. Determination of the molecular weights of MHV-specific intracellular RNAs. [^{32}P]Orthophosphate-labeled RNA was extracted from A59V- and JHMV-infected cells, precipitated with ethanol, and glyoxylated as described under Materials and Methods. Mengovirus, VSV, 18 and 28 S rRNA, adenovirus DNA, and *E. coli* 16 and 23 S rRNA were used as molecular weight markers. They were prepared for electrophoresis as described under Materials and Methods. A59V RNA, JHMV RNA, and the molecular weight markers were electrophoresed in parallel lanes of a 0.7% agarose slab gel. The positions of the *E. coli* rRNAs were determined by staining with acridine orange. The positions of the other nucleic acids were determined by autoradiography of the dried gel. A linear regression plot of the $\log_{10}$ of the molecular weights versus the distance migrated of the marker nucleic acids (■) was drawm. The positions of the MHV-specific RNAs are indicated by arrows.

ratios with one exception. The relative amount of RNA5 synthesized in JHMV-infected cells is about one-tenth of that synthesized in cells infected with A59V. This was a consistent finding in multiple experiments.

Kinetics of Appearance of MHV-Specific RNA Species

To determine if there is temporal regulation of MHV-specific RNA synthesis, replicate cultures of A59V, JHMV, or mock-infected cells were pulse labeled for 1 hr at hourly intervals and the intracellular RNA was extracted and analyzed by gel electrophoresis. The results for A59V-infected cells are shown in Fig. 4. Virus-specific RNA synthesis was barely detectable in infected cells labeled from 3 to 4 hpi. RNAs 4, 5, 6, and 7 were the only species present in detectable quantities at this time. At later times all A59V-specific RNA species were synthesized coordinately with respect to time. Virus-specific RNA synthesis was maximal in this experiment at 5–6 hr and then declined.

The results of a similar experiment with JHMV are shown in Fig. 5. Virus-specific RNA synthesis was first detected at 4–5 hpi. At this time all RNA species were present in detectable amounts although only RNAs 6 and 7 are visible in the photograph. At later times all of the JHMV-specific RNA species were easily detectable and were synthesized coordinately.

Affinity Chromatography of MHV-Specific RNA on Poly(U) Sepharose

RNA extracted from A59V, JHMV, and mock-infected cells was fractionated by chromatography over poly(U) Sepharose and then analyzed by gel electrophoresis (Fig. 6). The seven major MHV-specific RNAs were present in the material which bound to the poly(U) Sepharose (Pool 2). In addition, the two minor MHV-specific RNA species smaller than RNA 7 were also present in this pool. These RNAs are therefore presumably polyadenylated. That this is the case was confirmed by ribonuclease T_1 fingerprinting (see below). The RNA which did not bind to poly(U) Sepharose (Pool 1) consists of a heterogeneous mixture of RNA species. Discrete bands which coelectrophoresed with MHV-specific RNAs were present superimposed on a smear of heterogeneous material.

Ribonuclease T_1 Oligonucleotide Fingerprint Studies

The sum of the molecular weights of the six subgenomic RNA species exceeds the molecular weight of the genome by approximately 50%. To investigate this observation further, the seven major MHV-specific RNAs were purified and compared to each other and to virion RNA using the technique of ribonuclease T_1 fingerprinting.

The individual RNA species and virion RNA were purified from cells labeled with [^{32}P]orthophosphate as described under

Materials and Methods. The purified RNAs were digested with ribonuclease T_1 and the resulting oligonucleotides separated by two-dimensional gel electrophoresis. The results are shown in Figs. 7 and 8. Poly(A) tracts are seen as streaks in the upper left hand corner of the fingerprints of A59V and JHMV virion RNAs. This confirms the findings of others (Yogo *et al.*, 1977; Lai and Stohlman, 1978; Wege *et al.*, 1978) that the MHV genome is polyadenylated. The seven major A59V- and JHMV-specific intracellular RNAs are also polyadenylated.

A comparison of the fingerprints of the seven A59V-specific intracellular RNAs with each other and with A59V virion RNA reveals several things. The fingerprint of the largest intracellular RNA, species 1, is almost identical to that of virion RNA. There are, however, several oligonucleotides which are underrepresented in the fingerprint of RNA1 as compared to virion RNA (open triangles, Fig. 7, panel 1), as well as an oligonucleotide which is overrepresented in the fingerprints of RNA1 (closed triangle) and RNAs 6 and 7 (see below). All the oligonucleotides contained in RNA species 2 are present in RNA species 1 and are a subset of these oligonucleotides. Similarly, the fingerprint of RNA species 3 is a subset of the fingerprint of RNA species 2. An examination of the fingerprint of RNA species 4 reveals a new spot which is not present in any of the other A59V-specific RNAs (arrow in Fig. 7, panel 4). The remaining oligonucleotides observed in RNA species 4 are a subset of the larger RNA species. The fingerprint of RNA species 5 also contains a spot which is not present in any other RNA species (arrow in Fig. 7, panel 5). The remaining oligonucleotides in RNA species 5 are contained in the fingerprints of the larger RNA species. The fingerprint of RNA species 6 is a subset of that of RNA species 5. Similarly, all the oligonucleotides of RNA species 7 are contained in RNA species 6. However, one oligonucleotide (closed triangle, panels 6 and 7) is overrepresented in RNAs 6 and 7 when compared to virion RNA and RNAs 2, 3, 4, and 5. This oligonucleotide has the same

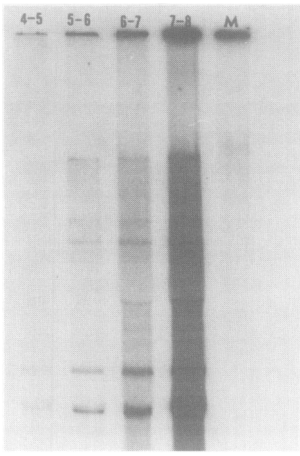

FIG. 5. The kinetics of JHMV-specific RNA synthesis. 17CL·1 cells were infected with JHMV or mock-infected, labeled at 1-hr intervals, and prepared for electrophoresis as described in Fig. 4. The times of labeling are indicated in the figure. Nucleic acids from mock-infected cells were run in lane M.

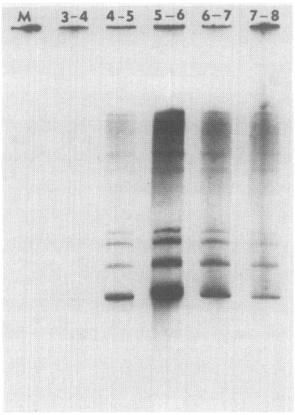

FIG. 4. The kinetics of A59V-specific RNA synthesis. 17CL·1 cells were infected with A59V or mock-infected, resuspended in DME2, and seeded in replicate 60-mm dishes and incubated at 37°. At 1-hr intervals individual dishes were removed from the incubator, media removed, washed twice with phosphate-free DME2, and then incubated for 15 min in phosphate-free DME2 containing 5 μg/ml actinomycin D. The cells were then labeled for 1 hr with 250 μCi/ml [^{32}P]orthophosphate and the cytoplasmic nucleic acids were then extracted and precipitated with ethanol. The RNA was collected by centrifugation, glyoxalated, and electrophoresed on a 1% agarose gel. The times of labeling are indicated in the figure. The lane containing RNA from mock-infected cells is labeled M.

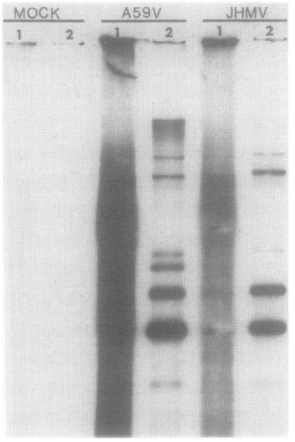

FIG. 6. Analysis of poly(A)-containing RNA. 17CL-1 cells were infected with A59V, JHMV, or mock-infected, seeded in 100-mm dishes in phosphate-free DME2, and labeled with [^{32}P]orthophosphate, 500 μCi/ml, in the presence of 5 μg/ml actinomycin D from 4 to 8 hr postinfection. Cytoplasmic extracts were prepared and the nucleic acids extracted and precipitated with ethanol. The nucleic acids were dissolved in a buffer containing 1 m*M* Tris, 360 m*M* NaCl,1 m*M* EDTA, 0.5% SDS, pH 7.4, chromatographed over poly(U) Sepharose as described by Wilt (1977), and fractionated into two pools. Pool 1 was not retained by the column; pool 2 contains the RNA which bound to the poly(U) Sepharose. Both pools were precipitated with ethanol after the addition of carrier tRNA, collected by centrifugation, glyoxalated, and analyzed by electrophoresis on a 0.8% agarose gel. The positions of rRNAs are marked by wire clips.

electrophoretic mobility as the oligonucleotide which is overrepresented in RNA1 and presumably is identical with it.

An analysis of the data obtained with JHMV (Fig. 8) reveals similar results. RNA1 is essentially identical to genomic RNA isolated from purified virions with the exception of an oligonucleotide which is underrepresented in RNA1 (open triangle). The six subgenomic JHMV-specific RNAs form a nested set as described above for the A59V specific RNA species, however, as for A59V, one oligonucleotide (closed triangle) is markedly overrepresented in RNAs 6 and 7 when compared to RNAs 2–5. The fingerprint of RNA species 4 contains a spot which is not present in any other JHMV-specific RNA (arrow in Fig. 8, panel 4). The reason for the overrepresentation of some oligonucleotides and the underrepresentation of others in these fingerprints is unclear at present. The spots which are underrepresented in RNA1 when compared to virion RNA disappear in RNA 2. These may represent oligonucleotides from the 5′ end of the genome which are selectively lost during the poly(U) Sepharose selection in the purification of RNA1. A more extensive analysis of the structure of MHV RNA should clarify this point.

A comparison of the fingerprints of A59V and JHMV virion RNAs shows only 10 to 20% sequence homology between these two viruses. This is in contrast to data obtained by hybridization analysis

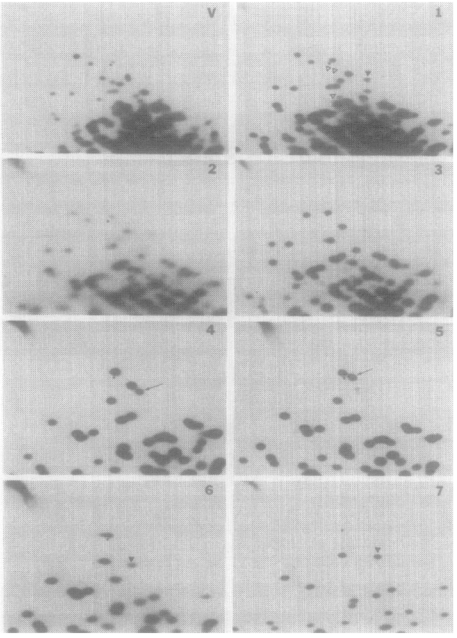

FIG. 7. Oligonucleotide fingerprints of A59V-specific RNA. A59V virion (panel V) and intracellular RNAs (panels 1–7) were purified as described under Materials and Methods. The purified RNAs were digested with RNAse T_1 and the resulting oligonucleotides separated by two-dimensional electrophoresis and autoradiographs prepared as described under Materials and Methods. Only the portion of the autoradiographs which contains oligonucleotides migrating more slowly than the xylene cyanol dye marker is shown. The positions of the bromphenol blue dye markers are idicated by asterisks. Unique spots are indicated by arrows.

which indicates 75% homology between the two genomes (Weiss and Leibowitz, in press) and the serologic cross reactions obtained with antisera to these viruses (Bond *et al.*, 1979). This discrepancy is not surprising since T_1 fingerprinting detects single base changes and will underestimate the degree of homology.

DISCUSSION

The data presented above indicate that MHV-infected cells synthesize at least seven virus-specific RNAs. The largest of these RNAs, RNA1, is nearly identical to virion RNA, as judged by oligonucleotide fingerprinting. The oligonucleotides of the six subgenomic RNAs, RNAs 2-7, are contained in the virion RNA. They are therefore of the same polarity as the virion RNA. Oligonucleotide fingerprints of these RNAs show them to make up a nested set in which any RNA contains the sequences present in any other smaller RNA plus additional sequences consistent with its larger size. Similar data have been obtained with another coronavirus, IBV (Stern and Kennedy, 1980a). A "Northern blot" analysis using representative and short 3′ specific cDNA probes indicates that the six subgenomic RNAs share sequences at their 3′ ends (Weiss and Leibowitz, in press). Furthermore, Stern and Kennedy (1980b) have established a 5′ to 3′ oligonucleotide spot order for the IBV genome. Their data show that the IBV subgenomic RNAs form a nested set with common 3′ ends. Taken together, these data suggest that the MHV-specific subgenomic RNAs map from the 3′ end of the genome in a similar manner to IBV. A possible model of this arrangement is shown in Fig. 9.

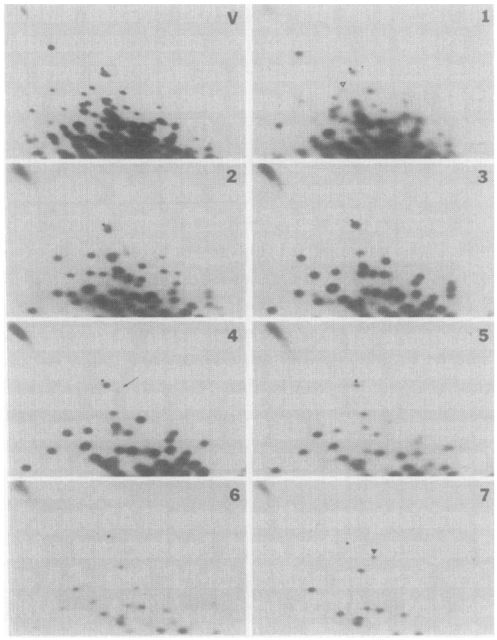

FIG. 8. Oligonucleotide fingerprints of JHMV-specific RNA. JHMV virion (panel V) and intracellular RNAs (panels 1-7) were purified as described under Materials and Methods. The purified RNAs were digested with RNAse T_1, the oligonucleotides separated by two-dimensional electrophoresis, and autoradiographs prepared. The positions of the bromphenol dye marker are shown by asterisks. Unique spots are indicated by arrows.

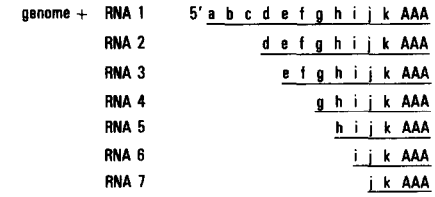

FIG. 9. A possible model of the sequence arrangement of MHV-specific RNAs.

It is unlikely that any of the six subgenomic RNAs are defective interfering RNAs. The stock virus used in these experiments was 3 passages (A59V) or 4 passages (JHMV) removed from cloning by limiting dilution (Robb and Bond, 1979b). The low m.o.i. used to grow virus stocks (10^{-4} PFU/cell) and to initiate infections in these experiments (0.1-0.15 PFU/cell) selects against the generation of defective interfering particles. Furthermore, after 10 serial undiluted passages of A59V and JHMV in 17CL·1 cells, the RNA gel pattern observed is unchanged from early passage virus (Leibowitz, unpublished data).

The six subgenomic RNAs are almost certainly mRNAs. They are polyadenylated, as expected of mRNAs, and Robb and Bond (1979) have shown that multiple MHV-specific RNA species spanning the

size range of the subgenomic RNAs 2-7 are present on polysomes of infected cells. Furthermore, Siddell *et al.* (1980) have recently demonstrated that polyadenylated RNA isolated from JHMV-infected cells can be translated *in vitro*. Using sucrose gradients they were able to partially resolve 17 and 19 S JHMV-specific RNA species. Their *in vitro* translation studies suggest that the 17 S RNA codes for the 60,000-dalton virion nucleocapsid protein (Sturman, 1977; Wege *et al.*, 1979), while the 19 S RNA codes for the 23,000-dalton virion glycoprotein (Sturman, 1977; Wege *et al.*, 1979). The relatively large amounts of these two RNAs and their sedimentation properties suggest that they correspond to RNAs 6 and 7. This has recently been confirmed by the *in vitro* translation of gel purified RNA (Leibowitz and Weiss, in press; van der Zeijst, in press). Van der Zeijst has also demonstrated that RNA3 codes for the 150,000-dalton protein described by Bond *et al.* (1979). This protein is the intracellular counterpart to the peplomer protein described by Sturman (1977) and Sturman and Holmes (1977). Of the five nonstructural proteins described by Bond *et al.* (1979), the 57,000- and 54,000-dalton proteins have been shown to have similar tryptic maps to the nucleocapsid protein (Leibowitz, unpublished data) and are presumably translated from the same mRNA. This leaves the three other nonstructural proteins described by Bond *et al.* (1979) and possibly a fourth nonstructural protein which has been described by Siddell *et al.* (1979) to be assigned to RNAs 2, 4, and 5. The *in vitro* translation of all the MHV-specific RNA species and appropriate characterization of the resulting products will be required for completion of these coding assignments.

Finally, the question arises about how the subgenomic RNAs are synthesized. Three mechanisms are possible. In the first mechanism, a full length negative strand copy is made from the positive strand genomic RNA. Each subgenomic RNA species is then separately transcribed from this negative strand template. This model would involve internal initiation of transcription. The subgenomic 26 S RNA of alphaviruses is generated in this manner (Brzeski and Kennedy, 1978). The second mechanism, again, involves the synthesis of a full length negative strand copy of the genome. This is then transcribed to a full length positive strand RNA. The subgenomic RNAs are then derived by nucleolytic cleavage of this full length positive strand RNA. In the third model, the subgenomic RNAs are each transcribed from subgenomic negative strand templates. These templates could originate by nucleolytic cleavage of genome length negative strand RNA or by internal termination of transcription. We have been unable to demonstrate any RNase-resistant, and therefore presumably double-stranded, RNAs in infected cells. Present data are not sufficient to distinguish between these three models or a combination of them. Further experiments using ultraviolet mapping of transcription initiation sites and the use of positive strand probes to identify negative strand templates may elucidate the mechanism of transcription of coronavirus-specific RNA.

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