MURINE CORONAVIRUS RNA

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INTRODUCTION

Coronaviruses are ubiquitous in nature and cause several diseases in infected hosts (Robb and Bond, 1979a). The genomic RNA of coronaviruses has been extensively studied in recent years (Lomneczi and Kennedy, 1977; Yogo et al., 1977; Lai and Stohlman, 1978; Wege et al., 1978; Macnaughton and Madge, 1978; Guy and Brian, 1979). These studies have shown that the coronavirus genome is a large, single stranded RNA which is polyadenylated and of positive polarity.

In contrast to the data obtained on virion RNA, there is a paucity of published data on intracellular coronavirus specific RNA. Robb and Bond (1979b) have studied mouse hepatitis virus (MHV) infected cells and found that RNA isolated from virus specific polysomes was heterogeneous in size (10-28S). Recently, Siddell et al. (1980) have shown that RNA of this size codes for two MHV structural proteins. Stern and Kennedy (1980) have identified six virus specific RNAs synthesized in cells infected with avian infectious bronchitis virus (IBV). These IBV specific RNAs have been fingerprinted and form a nested set.

We have been investigating the virus specific RNAs synthesized in cells infected with either MHV-A59 (A59V) or MHV-JHM (JHMV). JHMV is a highly neurotropic strain of MHV and produces a demyelinating encephalomyelitis in infected mice. A59V is weakly neurotropic. Both of these strains of MHV are able to initiate persistent

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infections in cell culture and in vivo. We have been investigating the replication of these viruses in the hope of eventually understanding how these viruses persist in infected mice.

MATERIALS AND METHODS

Cells

The origin and growth of the murine cell line 17CL-1 has been described previously (Sturman and Takemoto, 1972; Bond et al., 1979).

Virus

The origin and growth of A59V and JHMV virus stocks has been described (Robb and Bond, 1979b). Cells were infected in suspension and virus adsorption was at 37°C for 30 minutes. Following adsorption the cells were centrifuged, resuspended in Dulbecco's Modified Eagle's Medium with 2% fetal bovine serum (DME-2) prewarmed to 37°C 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°C. For most experiments utilizing ^{32}P-orthophosphate as label, the cells were resuspended and plated in phosphate free DME-2.

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 minutes, vortexed again and the nuclei removed by centrifugation at 1,500 g for 2 minutes. 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°C for five minutes and at room temperature for an additional 25 minutes. The RNA was then extracted with phenol chloroform and precipitated with 3 volumes of ethanol.

Preparation of MHV Virion RNA

17CL-1 cells were infected with A59V or JHMV in phosphate free DME-2 as described above. Following virus adsorption the cells were plated in 100 or 150 mm culture dishes and incubated at 33°C. At 4 hours post infection (h.p.i.) ^{32}P-orthophosphate was added to a concentration of 100-1,000 µci/ml. At 16-18 h.p.i. the cell associated virus was released by two cycles of freeze thawing and the resulting lysate clarifeid by centrifugation at 10,000 g for 30 minutes at 4°C. Virus was concentrated by centrifugation for 60

minutes 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 sonciation 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 minutes 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 hours. 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/cc. The virus was diluted with MSE buffer, pelleted at 45,000 rpm for 30 minutes 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 minutes at 20°C in the SW50.1 rotor. The gradient was fractionated and the peak of virion RNA 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

Analytical electrophoresis. Agarose gel electrophoresis following glyoxal denaturation was essentially as previously described (McMaster and Carmichael, 1977). Samples were electrophoresed at 100 V for 4 hours 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.

Preparative electrophoresis. Labeled intracellular RNA was extracted from 1.2 x 10^8 to 2.0 x 10^8 cells and the poly(A) containing RNA species selected by affinity chromatography. The RNA was denatured with 10 mM methyl mercuric hydroxide and electrophoresed in agarose gels containing 5 mM methyl mercuric hydroxide (Bailey and Davidson, 1976). Electrophoresis was at 100 volts for 6 hours in a horizontal gel containing either 0.8% agarose or 1% low melting agarose, depending on the method used to elute the RNA from the gel (See below.). 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).

RNA Elution

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 wit a flamed scalpel. For T_1 fingerprint studies low melting point agarose gels were used and RNA was recovered from gels by melting the agarose at 70°C for 5 minutes in the presence of Tris acetate buffer (10 mM Tris, 20 mM sodium acetate, 5 mM EDTA), 1% 2-mercaptoethanol. Five ml of a hydroxylapatite (HA) slurry, prewarmed to 37°C, was added and the HA was collected by centrifugation and washed twice with Tris acetate buffer (pH 7.3) in a 37°C warm room to remove the liquified agarose. The HA was resuspended in 5 ml of Tris acetate buffer and transferred to disposable columns. The RNA was eluted from HA columns by two washes of 1.0 ml of 0.4 M sodium phosphate, 1 mM EDTA (pH 7.0) which were forced from the columns by centrifugation at 1,000 g for 10 minutes. The phosphate eluates were pooled and the RNA was precipitated with CTAB as described by Stern and Kennedy (1980). The CTAB precipitates were dissolved in 0.2 ml of 50 mM Tris, pH 7.4, containing 1M NaCl and 1 mM EDTA and the RNA was precipitated with ethanol.

RNA to be used in translation studies was electrophoresed in 0.8% standard melting point agarose gels. The RNA species were located as above, agarose strips were excised from the gel with a scalpel and ground up in a tissue homogenizer containing a high salt buffer (10 mM Tris, 0.5 M NaCl, 1 mM EDTA, 1% SDS, 1% 2-mercapto-ethanol). This material was extracted overnight at 4°C on a rocker platform with an equal volume of phenol. The phases were separated by centrifugation, the aqueous phase was re-extracted and the RNA precipitated with ethanol in the presence of rabbit liver tRNA.

Ribonuclease T_1 Fingerprinting

The RNA was digested with 10 µl of ribonuclease T_1 (1 mg/ml) in 10 mM Tris, pH 7.6, at 37°C for 30 minutes. Ten µl 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

resistant oligonucleotides were separated by two dimensional polyacrylamide gel electrophoresis as described by Stern and Kennedy (1980) with the following modification. After electrophoresis in the first dimension, the gel strips were washed for 25 minutes with two changes of 100 mM Tris borate buffer containing 2.5 mM 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 with intensifying screens (Laskey and Mills, 1977).

In Vitro Translation

A rabbit reticulocyte lysate system treated with micrococcal nuclease (Pelham and Jackson, 1976) was used with ^{35}S-methionine as the label.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970).

Tryptic Peptide Map Studies

Tryptic peptide map studies were performed as described previously (Gibson, 1974).

RESULTS

Identification of MHV Specific Polyadenylated RNA species

17CL-1 cells were infected with A59V, JHMV or mock infected and labeled with ^{32}P-orthophosphate from 4-8 h.p.i. in the presence of actinomycin D. The cytoplasmic RNA was extracted and separated into polyadenylated (poly(A)$^+$) and non-polyadenylated (poly(A)$^-$) classes by chromatography over poly(U) Sepharose. These RNAs were denatured with glyoxal and then analyzed by agarose gel electrophoresis. Seven MHV specific poly(A)$^+$ RNAs were reproducibly present in infected cells (Fig. 1). These RNAs have been designated RNAs 1-7, in decreasing order of size. In addition, we have occasionally observed two additional RNA species migrating faster than RNA 7 and minor RNA species migrating between RNAs 1 and 2 and RNAs 3 and 4. These minor species have not been studied extensively as yet.

The largest MHV specific RNA, RNA 1, comigrates with virion RNA and represents the intracellular form of the genome. In contrast to the differences observed between A59V and JHMV specific proteins (Bond et al., 1979), A59V and JHMV specific RNAs co-electrophorese in agarose gels.

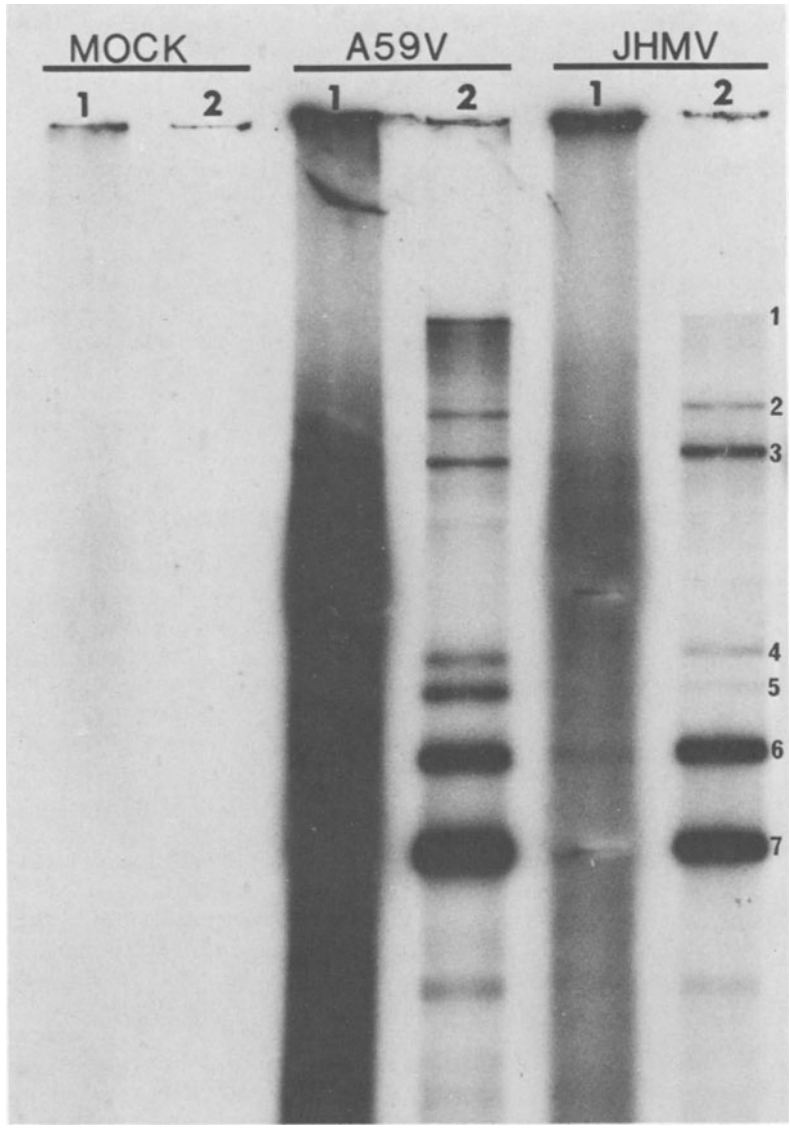

Fig. 1. The identification of MHV specific poly(A)$^+$ RNA species. 17CL-1 cells were infected with A59V, JHMV or mock infected, and labeled with ^{32}P-orthophosphate in the presence of 5 µg/ml actinomycin D from 4-8 hours post infection. Cytoplasmic nucleic acids were extracted, precipitated and chromatographed over poly(U) Sepharose as described by Wilt (1977). The poly(A)$^-$ RNA(1) and poly(A)$^+$ RNA (2) species were glyoxylated and electrophoresed on a 0.8% agarose gel.

The poly(A)$^-$ MHV specific RNA is a heterogeneous mixture. Discrete bands which comigrate with poly(A)$^+$ MHV specific RNA are superimposed on a smear of heterogeneous material.

The sizes of MHV specific RNAs 1-7 were determined by agarose gel electrophoresis with appropriate molecular weight markers (Table 1). A linear-log relationship was obtained over a molecular weight range of 5.5×10^5 to 1.15×10^7 daltons.

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 percent. To further investigate this observation, the 7 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, digested with ribonuclease T_1 and the resulting oligonucleotides were separated by two dimensional gel electrophoresis. The results are shown in Figures 2 and 3. 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.

Table 1. The Molecular Weights of MHV Specific RNA

RNA Species	Mol. wt.[a]
Virion RNA	6.1×10^6
RNA 1	6.1×10^6
RNA 2	3.4×10^6
RNA 3	2.6×10^6
RNA 4	1.2×10^6
RNA 5	1.08×10^6
RNA 6	8.5×10^5
RNA 7	6.3×10^5

[a]Molecular weights were determined by electrophoresis after glyoxal denaturation in parallel to the following markers. E. coli rRNA, 17CL-1 cell rRNA, mengovirus RNA, VSV RNA, adenovirus type 2 DNA.

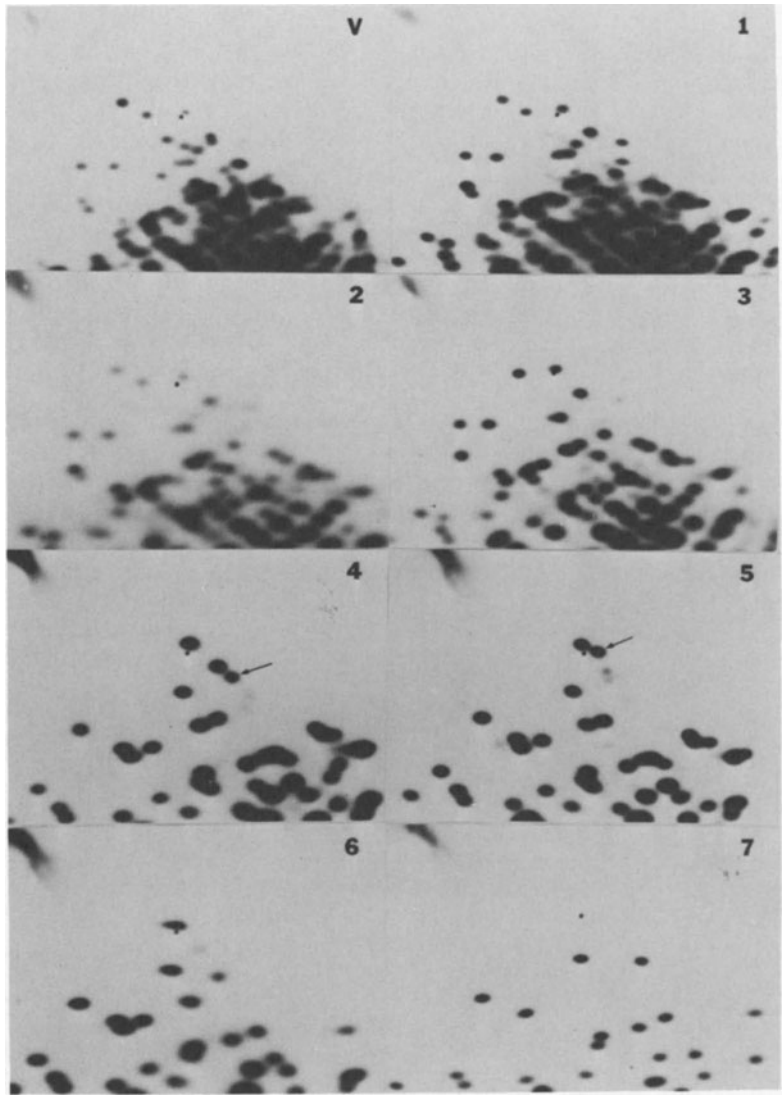

Fig. 2. Oligonucleotide fingerprints of A59V specific RNA. A59V virion (panel V) and intracellular RNAs (panels 1-7) were purified, digested with RNase T_1 and the resulting oligonucleotides separated as described in Materials and Methods. Only the portion of the autoradiographs which contains oligonucleotides migrating more slowly than the bromphenol blue dye markers are shown. The positions of the xylene cyanol dye markers are indicated by asterisks. Unique spots are indicated by arrows.

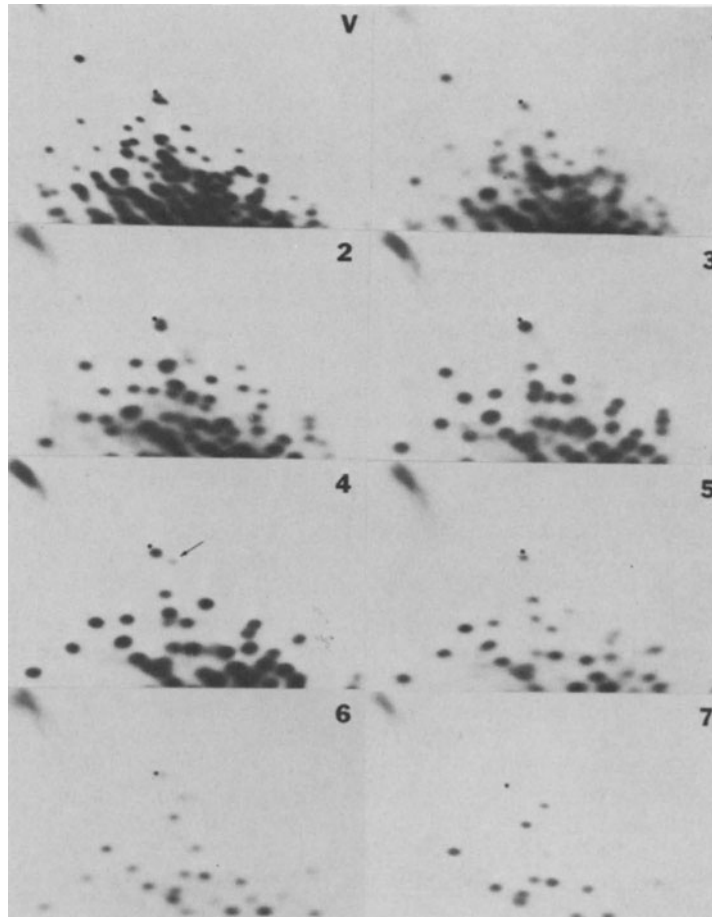

Fig. 3. Oligonucleotide fingerprints of JHMV specific RNA. JHMV virion (panel V) and intracellular RNAs (panels 1-7) were purified and oligonucleotide fingerprints prepared as in Fig. 2. The positions of the xylene cyanol dye marker are shown by asterisks. Unique spots are indicated by arrows.

A comparison of the fingerprints of the seven A59V specific intracellular RNAs with each other and with A59V virion RNA (Fig. 2) reveals several things. The fingerprint of the largest intracellular RNA, species 1, is essentially identical to that of virion RNA. 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. 2, 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. 2, 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.

Several conclusions can be drawn from this data. Firstly, the seven intracellular A59V specific RNAs are indeed virus specific and are of the same polarity as virion RNA. Secondly, these RNAs form a nested set with each succeeding larger RNA containing additional sequences.

An analysis of the data obtained with JHMV (Fig. 3) reveals similar results. RNA 1 is essentially identical to genomic RNA isolated from purified virions. The six subgenomic JHMV specific RNAs form a nested set as described above for the A59V specific RNA species. The fingerprint of RNA species 4 contains a spot which is not present in any other JHMV specific RNA (arrow in Fig. 3, panel 4).

The significance of the anomalous spots observed in the fingerprints of A59V RNAs 4 and 5 and JHMV RNA 4 is unknown. If these new oligonucleotides are at the 5' termini of RNAs 4 and 5 they could be truncated derivatives of oligonucleotides present in the fingerprints of A59V and JHMV virion RNA. Alternatively, these new spots are adjacent to spots seen in virion RNA and it is possible that they represent minor modifications, such as methylation, of these oligonucleotides.

In Vitro Translation of Intracellular A59V Specific RNA

The seven subgenomic poly(A)$^+$ RNAs were purified from A59V infected cells by agarose gel electrophoresis as described in Materials and Methods. These RNAs were added to the nuclease treated reticulocyte lysate *in vitro* translation system described by Pelham and Jackson (1976). The products of these translation experiments were analyzed by polyacrylamide gel electrophoresis. The results of one of these experiments are shown in Figure 4. *In vitro* translation of RNA 7 results in the synthesis of two virus specific polypeptides; a minor product which co-electrophoreses with nucleocapsid protein and a major product which migrates slightly faster than the nucleocapsid protein. Analysis of tryptic digests of these two polypeptides and the intracellular nucleocapsid protein by 2-dimensional chromatography and electrophoresis shows that they have identical methionine containing tryptic peptides (Fig. 5). Translation of RNA 6 results in the synthesis of two polypeptides. The larger of these polypeptides comigrates with the major translation product of RNA 7 and has

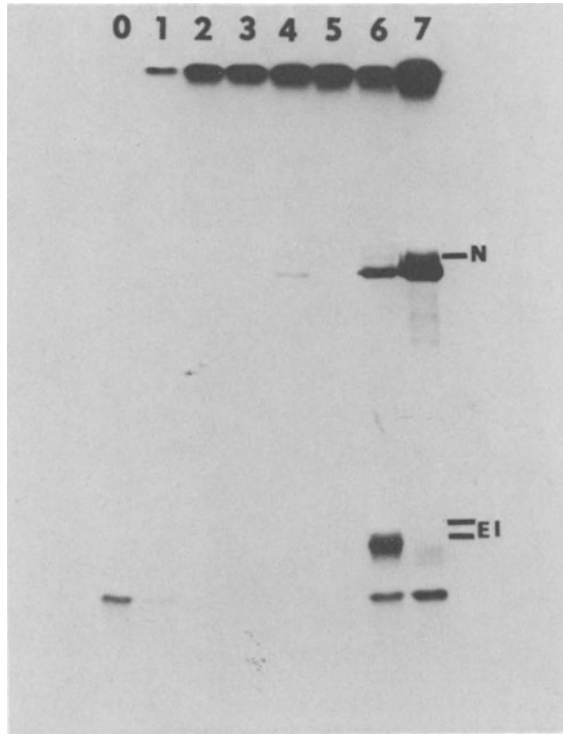

Fig. 4. The translation of A59V specific RNAs. Intracellular RNAs were purified and translated as described in Materials and Methods. The products of the translation were analyzed by polyacrylamide gel electrophoresis on a 10% gel. Lane 0 is the analysis of a translation of a lysate with no added RNA. Lanes 1-7 are the analyses of translations of purified A59V specific RNAs 1-7 respectively. The positions of A59 nucleocapsid (N) and E1 proteins are indicated.

an identical ^{35}S-methionine tryptic peptide map (Fig. 5). The smaller translation product of RNA 6 co-electrophoreses with the lower molecular weight form of the E1 protein. The ^{35}S-methionine tryptic peptide map of this protein and the A59V E1 protein are identical (Fig. 6). Translation of the larger, less abundant RNAs demonstrated one additional product. A longer exposure of the gel shown in Figure 4 demonstrates a minor band with a molecular weight of approximately 35 000 daltons in the translations of RNAs 4, 3 and 2 (date not shown). This material is currently being analyzed further to determine if it corresponds to the previously described non-structural A59V protein of the same molecular weight (Bond et al., 1979).

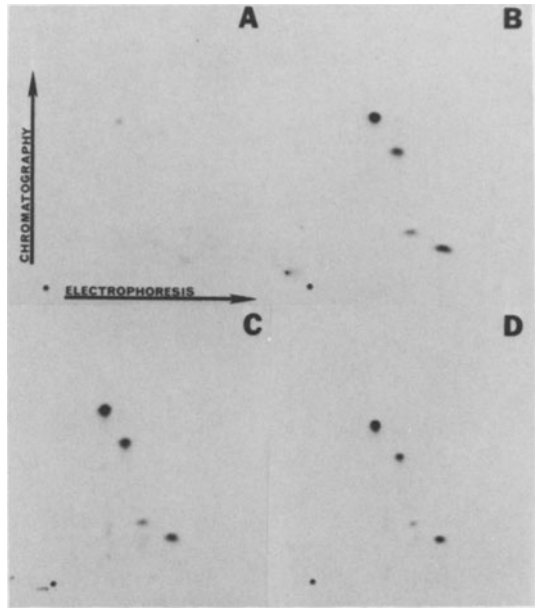

Fig. 5. The tryptic maps of in vitro synthesized nucleocapsid protein. The ^{35}S-methionine labeled in vitro translation products of RNA 7 were purified by polyacrylamide gel electrophoresis, digested with trypsin and analyzed as described by Gibson (1974). The tryptic maps of the major and minor translation products (See text.) are shown in panels B and A, respectively. The translation product of RNA 6 which co-electrophoresed with the major translation product of RNA 7 (see text) was similarly analyzed (panel C), as was ^{35}S-methionine labeled A59V nucleocapsid protein purified from infected cells (panel D). The origins are indicated by asterisks.

Translation of Virion RNA

Following virus purification, intact A59V and JHMV virion RNA was further purified by sedimentation on sucrose gradients. This RNA was translated in the reticulocyte lysate system and the products were analyzed by gel electrophoresis. As shown in Figure 7, the translation of four different preparations of virion RNA resulted in three polypeptides of over 200,000 daltons molecular weight. These polypeptides do not co-electrophorese with any known MHV specific proteins. Experiments to demonstrate these polypeptides in infected cells have been unsuccessful to date. Analysis of these three polypeptides by the limited proteolysis method of Cleveland et al.

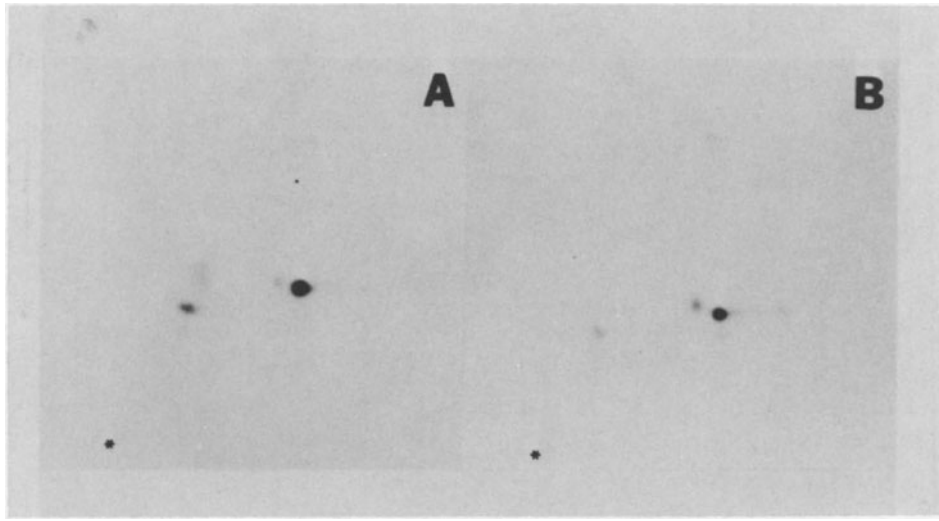

Fig. 6. The tryptic map of in vitro synthesized E1 protein. The translation product of RNA 6 which co-electrophoreses with E1 protein (panel A) and E1 protein prepared from purified A59V (panel B) were analyzed as described in Figure 5.

(1977) shows them to be structurally related to each other (Data not shown.).

DISCUSSION

The data presented above indicate that MHV infected cells synthesize at least seven virus specific RNAs. The largest of these RNAs, RNA 1, is identical to virion RNA as judged by oligonucleotide fingerprinting. The oligonucleotides of the six subgenomic RNAs, RNA 2-6, 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, 1980). A "Northern blot" analysis using representative and short 3' specific c-DNA probes indicates that the six subgenomic RNA's share sequences at their 3' ends (Weiss and Leibowitz, this symposium). Furthermore, Stern and Kennedy (this symposium) 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 Figure 8.

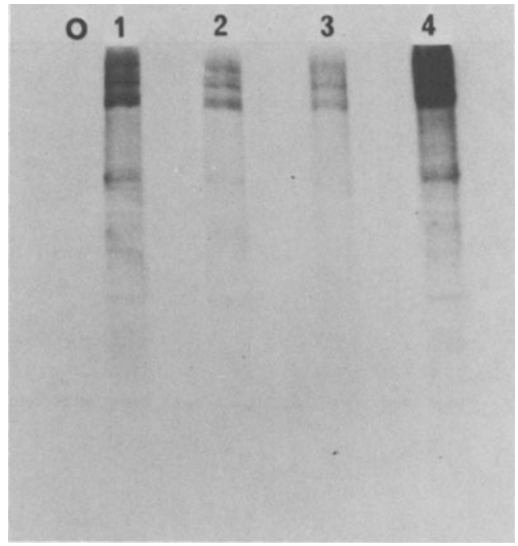

Fig. 7. In vitro translation of virion RNA. Virion RNA was purified and translated in vitro as described in Materials and Methods. The translation products were analyzed by polyacrylamide gel electrophoresis. Lane 0 is the analysis of a translation with no added RNA; lanes 1, 3 and 4, with three different preparations of A59V RNA; lane 2 with JHMV RNA.

genome +	RNA 1	5′ a b c d e f g h i j k AAA 3′
	RNA 2	d e f g h i j k AAA
	RNA 3	e f g h i j k AAA
	RNA 4	g h i j k AAA
	RNA 5	h i j k AAA
	RNA 6	i j k AAA
	RNA 7	j k AAA

Fig. 8. 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 to 0.15 PFU/cell) select against the

generation of defective interfering particles. Furthermore, after ten 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).

At least two, and probably all six, subgenomic RNAs are mRNAs. They are polyadenylated, as expected of mRNAs, and Robb and Bond (1979b) 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. The data presented in this paper demonstrate that RNA 7 codes for the nucleocapsid protein. The small difference in migration of the major in vitro translation product of this RNA and the in vivo nucleocapsid protein could perhaps be due to phosphorylation, as the virion nucleocapsid had been shown to be phosphorylated (Stohlman and Lai, 1979). Translation of RNA 6 results in two products, the E1 protein and the nucleocapsid protein. This result is consistent with the proposed physical arrangement of the genome shown in Figure 8. The E1 protein is encoded in the sequences at the 5' end of RNA 6 (designated j and k) coding for the nucleocapsid. The data we have obtained does not distinguish between the synthesis of the nucleocapsid protein from RNA 6 being due to internal initiation of protein synthesis or degradation of the RNA during the translation resulting in the exposure of an internal initiation site.

Translation of purified virion RNA results in the synthesis of three structurally related polypeptides greater than 200,000 daltons molecular weight. The coding capacity of the 5' end of the genome which is not present in the subgenomic mRNAs (sequences a, b and c in Figure 8) is approximately 270,000 daltons and will accommodate these polypeptides. These polypeptides are likely to function as the intracellular MHV specific RNA polymerase by analogy to other positive stranded RNA viruses. Further work is needed to demonstrate these polypeptides in infected cells or in an in vitro MHV specific RNA polymerase system.

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