

Genomic RNA of the Murine Coronavirus JHM

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SUMMARY

Genomic RNA extracted from the purified murine coronavirus JHM sediments between 52S and 54S in aqueous sucrose gradients. The RNA is single-stranded and has an apparent mol. wt. of 5.4 to 6.5×10^6 , as determined by electrophoresis in polyacrylamide agarose gels of different concentrations. The presence of polyadenylate sequences in the RNA is demonstrated by binding to oligo-(dT) cellulose and digestion with ribonucleases A and T₁. The purified RNA does not dissociate into subunits at high temperatures or in high concentrations of DMSO and is infectious.

INTRODUCTION

Coronaviruses are found in many animal species including man and are associated with a great variety of diseases. They cause respiratory and enteric diseases in humans, bronchitis in birds, transmissible gastroenteritis and encephalitis in pigs, diarrhoea in calves and dogs, peritonitis in cats and both demyelinating encephalitis and hepatitis in rodents (McIntosh, 1974; Tyrrell *et al.* 1975). Despite this great variety of diseases and widespread occurrence in nature, little biochemical and biological information about these viruses is available and evidence for including these viruses into one group is mainly based on morphological and serological characteristics.

Information on coronavirus genomic RNA is available for avian bronchitis virus, two porcine strains, one mouse strain and one human coronavirus. These data are partly controversial and incomplete. The genomic RNA of avian bronchitis virus (IBV) consists of a single stranded RNA, which is polyadenylated and infectious (Lomniczi, 1977; Schochetman *et al.* 1977). Evidence for polyadenylation was also recently reported for a mouse hepatitis virus (Yogo *et al.* 1977). Estimates of the mol. wt. of genomic RNA were published for human and avian strains, and varied between 5.5 to 9×10^6 (Tannock, 1973; Watkins *et al.* 1975; Lomniczi & Kennedy, 1977; MacNaughton & Madge, 1977; Schochetman *et al.* 1977; Tannock & Hierholzer, 1977). Moreover, evidence was presented that the RNA of porcine viruses is not a continuous strand but consists of subunits analogous to tumour virus RNA (Garwes *et al.* 1975).

Our interest in the murine coronavirus strain JHM emerged from the search for an animal model to study chronic and subacute diseases of the central nervous system (CNS) associated with virus infections. Coronavirus JHM is a neurotropic mouse hepatitis virus, which induces these CNS changes (Nagashima *et al.* 1978).

However, to analyse the virus host relationship in these diseases it is essential to have detailed information on the structure and molecular biology of this virus. The present report characterizes the genomic RNA of the murine coronavirus JHM.

METHODS

Cells and viruses. JHM virus was originally obtained from Dr L. Weiner, Johns Hopkins University, Baltimore, U.S.A., as a mouse brain suspension. It was adapted to grow on L929 cells and Sac(-) cells. L929 originated from the American Type Culture Collection. Sac(-) cells are a murine rhabdomyosarcoma line obtained from Dr Mussgay, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany. Both lines were maintained in minimal essential medium with 5% foetal calf serum.

Virus growth. JHM virus was plaque-purified and grown on Sac(-) cells. L cells were only used for virus titrations and not for virus propagation, since infection with JHM virus leads to the induction of endogenous C-type particles in this cell line. This effect was demonstrated by electron microscopy, radioimmunoassay and tests for reverse transcriptase. Sac(-) cells did not show induction of C-type particles.

Monolayers in Roux-bottles were infected with 0.05 p.f.u./cell by adsorption for 1 h at 37 °C. Growth medium consisted of minimal essential medium containing double the amount of amino acids and 5% foetal calf serum. The virus was harvested 20 to 24 h later, when more than 75% of the cells formed syncytia. The medium of infected cell-cultures contained between 5×10^6 to 5×10^7 p.f.u./ml.

Radioactive labelling of virus material. Twelve hours after infection the medium was removed and replaced by minimal essential medium with 5% foetal calf serum containing 10 μ Ci/ml 3 H-uridine or the same amount of 3 H-adenosine. Until harvest the cells were incubated on a rocker platform at 37 °C.

Virus purification. All procedures were performed at 4 °C. The cells were scraped from the glass and separated by centrifugation from the virus containing medium. After addition of 0.5 M-NaCl the virus was precipitated by addition of polyethyleneglycol 6000 (PEG) to a final concentration of 10% (w/v). The buffer used for dissolving polyethyleneglycol (30%, w/v) and making sucrose solutions consisted of 0.01 M-tris-HCl (pH 6.5), 0.1 M-NaCl, 0.001 M-EDTA (NTE buffer). After stirring for 10 min the virus was pelleted in a Sorvall GSA rotor at 10000 g for 30 min. The precipitated virus was homogenized in NTE, clarified by centrifugation for 10 min at 3000 rev/min and the virus was pelleted through a gradient consisting of 22 ml 5 to 20% (w/v) sucrose underlaid with a cushion of 4 ml 60% (w/w), sucrose. The gradient was centrifuged for 2 h at 23000 rev/min in a Beckman SW 27 rotor. The virus-containing interface was harvested, diluted with NTE to less than 20% sucrose and loaded on a linear gradient consisting of 20 to 60% (w/w) sucrose.

The virus was centrifuged to equilibrium at 20000 rev/min for 12 h in a SW 27 rotor. After harvest the virus band was diluted again in NTE and pelleted at 25000 rev/min for 90 min. The pelleted material was immediately used for extraction of RNA.

Extraction of 3 H-uridine and 3 H-adenosine labelled RNA. RNA was extracted from purified virus either by treatment with SDS or by extraction with phenol. For SDS extraction pellets of purified virus were incubated in NTE (pH 7.4) containing 2% SDS (w/v). After 10 min at 37 °C and 3 min at 56 °C the lysed virus was loaded on to an aqueous gradient (15 to 30% (w/w) in NTE, pH 7.4, with 0.5% SDS, 16 ml, using a SW 27.1 rotor) and centrifuged for 14 h at 20000 rev/min, 20 °C. Virus RNA was recovered from the gradient fractions by ethanol precipitation. Alternatively, RNA was extracted from the virus by treatment with phenol-chloroform-isoamylalcohol (50:50:1) in the presence of SDS at room temperature (pH 8.7) as described by Aviv & Leder (1972). Ribosomal RNA from Sac(-) cells and RNA from purified vesicular stomatitis virus (VSV) Indiana strain were also extracted by lysis with SDS and separated by velocity sedimentation. RNA from Rous sarcoma virus (RSV) strain

PR-C labelled with ^{32}P or ^{14}C was a generous gift from Dr W. Rhode, Institut für Virologie, Giessen, W. Germany.

Velocity sedimentation. Extracted RNA was further purified and analysed in 15 to 30% sucrose gradients as described above. The distribution of virus RNA within the gradient was determined by scintillation counting of trichloroacetic acid insoluble counts from each fraction collected on to Whatman GF/C filters. Markers made from ribosomes, VSV and RSV were sedimented both in parallel and in combinations with JHM virus RNA.

Polyacrylamide agarose gel electrophoresis. Purified RNA was analysed in tube gels containing 1.6 or 2.0% (w/v) polyacrylamide solidified with 0.64% agarose, according to the procedures of Loening (1969). The buffer (NET, pH 7.8) consisted of 30 mM- NaH_2PO_4 , 36 mM-tris-HCl, 1 mM-EDTA and 0.1% SDS. Gels were electrophoresed in the cold with 90 V and 7 mA/12 cm gel for 2.5 to 3 h. Marker RNAs (rRNA, VSV and RSV RNA) were electrophoresed both in combination with JHM RNA and in parallel tubes. Gels were processed by slicing and the radioactivity was determined by scintillation counting after solubilization with solunene.

Assay of polyadenylated sequences. Oligo-(dT) cellulose columns were made according to the method of Aviv & Leder (1972). Pellets of purified RNA were dissolved in binding buffer (0.01 M-tris-HCl, pH 7.4; 0.5 M-NaCl; 0.001 M-EDTA and 0.1% SDS). Elution of bound RNA was performed with the same buffer without NaCl. Both bound and unbound RNA were recovered by ethanol precipitation.

For digestion with ribonucleases purified genomic RNA (5 to 10 μg) was dissolved in NTE, pH 7.4, and 25 μg yeast RNA was added. The final mixture contained in 200 μl , 20 μg of ribonuclease A and 450 units ribonuclease T₁ (Boehringer, Mannheim). After 30 min at 37 °C the mixture was cooled in ice and the trichloroacetic acid precipitable material sampled on Whatman GF/C filters for scintillation counting. Isolation of poly A sequences was done by adding 2% (w/v) SDS to the digested material. The digested RNA was loaded on a gradient (5 to 20% sucrose (w/w) in NTE, pH 7.4, with 0.5% SDS) and spun for 12 h at 40000 rev/min in a Beckman SW41 rotor. *Escherichia coli* tRNA was added as marker. The radioactivity was determined by scintillation counting after precipitation of individual fractions with trichloroacetic acid.

Treatment of JHM RNA by heat and DMSO. Extracted RNA purified by velocity sedimentation was dissolved in NET buffer pH 7.8 and heated at 80 or 100 °C for 3 min in sealed ampoules and cooled in melting ice. Control RSV RNA was treated in the same manner. The treated RNAs were analysed by gel electrophoresis. These conditions are sufficient to denature RSV virion RNA completely into subunits of approximately 35S. DMSO treatment was performed by adding 20 vol. DMSO to RNA dissolved in NET buffer and the mixture was incubated at room temperature for 30 min and cooled in ice as described by Duesberg (1968). The RNA was recovered by ethanol precipitation and analysed as described above.

Infectivity of JHM RNA. RNA extracted by both lysis with SDS or phenol from purified virus was twice purified on velocity gradients and recovered by ethanol precipitation. The final pellet was dissolved in autoclaved double distilled water and mixed with an equal vol. of 1.4 M- MgSO_4 as described by Wecker *et al.* (1962). Monolayers of Sac(-) cells (cluster plates, 2 cm²/well, 2×10^5 cells/well) which had been pre-incubated with saline containing 0.02 M- MgSO_4 for 15 min at 37 °C were overlaid with different concentrations of virus RNA (0.2 ml/well) mixed with DEAE-dextran (0.5 mg/ml). After 25 min adsorption at room temperature the cells were washed with saline and minimal essential medium containing 5%

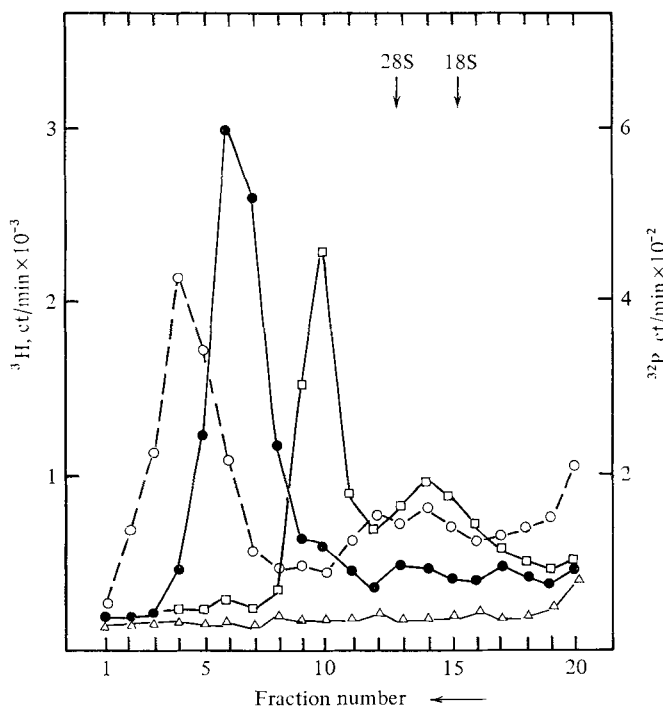


Fig. 1. Sedimentation of extracted JHM RNA in sucrose gradients. Virus labelled with ^3H -uridine was purified and the RNA extracted by lysis with SDS. The RNA was centrifuged through a 16 ml 15 to 30% (w/w) sucrose gradient in NTE containing 0.5% SDS at 20000 rev/min for 14 h at 20 °C. Markers of ribosomal RNA and ^{32}P -RSV were centrifuged in the same gradient. ^3H -VSV RNA mixed with ribosomal RNA and JHM RNA in a parallel gradient. ●—●, JHM RNA; ○—○, ^{32}P -RSV RNA; □—□, ^3H -VSV RNA; △—△, JHM RNA, recovered from the 52 to 54S region and treated with ribonuclease A before velocity sedimentation in a parallel gradient.

foetal calf serum was added. The cells were monitored for c.p.e. for 3 days and a negative result was only accepted after two blind passages.

Materials. The following radiochemicals were purchased from the Radiochemical Centre, Amersham, U.K.: 5- ^3H -uridine (20 to 30 Ci/mmol), 2- ^3H -adenosine (24 Ci/mmol). *Escherichia coli* tRNA, pancreatic ribonuclease A (40 units/mg), ribonuclease T1 (5000 units/mg) and polyuridylic acid were supplied by Boehringer Mannheim. Oligo(dT)-cellulose type 7 was bought from P.L. Biochemicals Inc.; acrylamide and polyethyleneglycol 6000 came from Serva. Agarose was obtained from Litex, Denmark. Soluene was obtained from Packard.

RESULTS

Size estimation of JHM virus RNA

RNA extracted by SDS-lysis or phenol from purified virus (Fig. 1) sedimented in sucrose gradients as a single homogeneous peak between 52S and 54S. The value was obtained by comparison with labelled RNA from ribosomes, RSV and VSV, and correlates to an apparent mol. wt. of 6.2×10^6 to 6.7×10^6 . Both RNA extracted with phenol and by lysis with SDS alone had the same S value. The electrophoretic migration of JHM virus RNA in polyacrylamide-agarose gels was influenced by the polyacrylamide concentration of the gel system. In gels containing 1.6% polyacrylamide JHM virus RNA migrated as a single, sharp

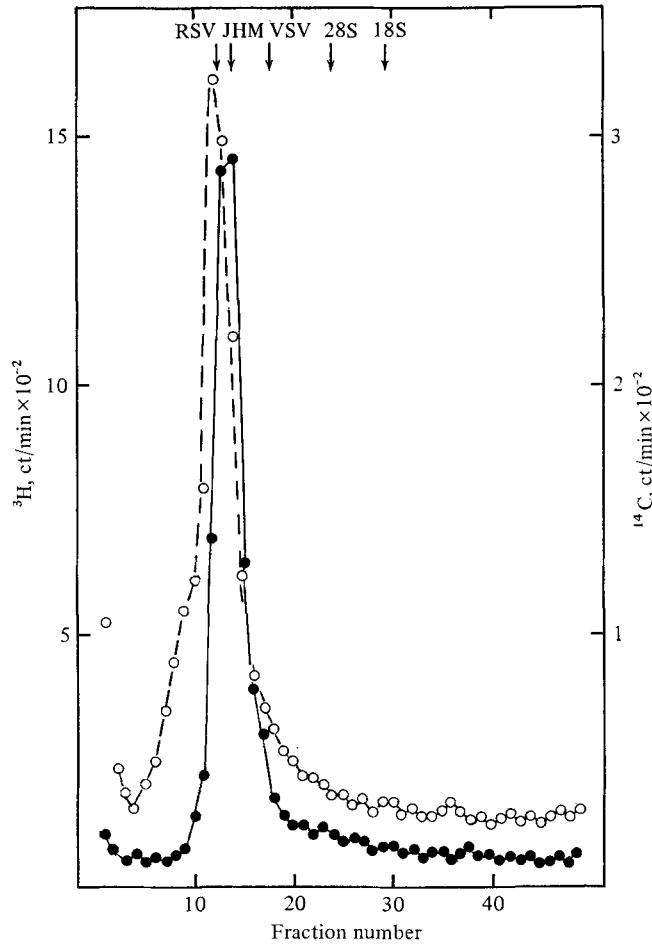


Fig. 2. Electrophoresis of JHM RNA in 1.6% polyacrylamide-agarose gels. ^{14}C -RSV RNA (\circ --- \circ) was mixed with purified ^3H -JHM RNA (\bullet — \bullet) and ribosomal marker RNAs (determined by measurement of absorbance). ^3H -VSV RNA was electrophoresed in combination with ^{14}C -RSV RNA, ribosomal markers and JHM in parallel gels. The mol. wt. for the marker RNAs were: 1.75×10^6 and 0.75×10^6 for 28S and 18S ribosomal RNA (Loening, 1968); 3.8×10^6 for VSV RNA (Repik & Bishop, 1973) and 7.6×10^6 for RSV RNA (King, 1976).

peak close to RSV RNA (Fig. 2). The apparent mol. wt. calculated relative to the marker RNAs (ribosomal RNA, VSV RNA and RSV RNA) is $6.5 \times 10^6 \pm 0.3$ (range of six determinations). In gels containing 2% polyacrylamide the JHM virus RNA migrated close to VSV RNA (Fig. 3). The apparent mol. wt. calculated from these gels (four determinations) is 5.4×10^6 . JHM virus RNA was electrophoresed both in combination with RSV RNA and in parallel gels.

To confirm the single-strandedness of the genomic RNA the material from the 52 to 54S region was recovered by ethanol precipitation. The RNA was dissolved in NTE buffer and digested with ribonuclease A ($100 \mu\text{g}/\text{ml}$, 30 min, 37°C). The digested material was entirely susceptible to ribonuclease, as judged by velocity sedimentation analysis (Fig. 1).

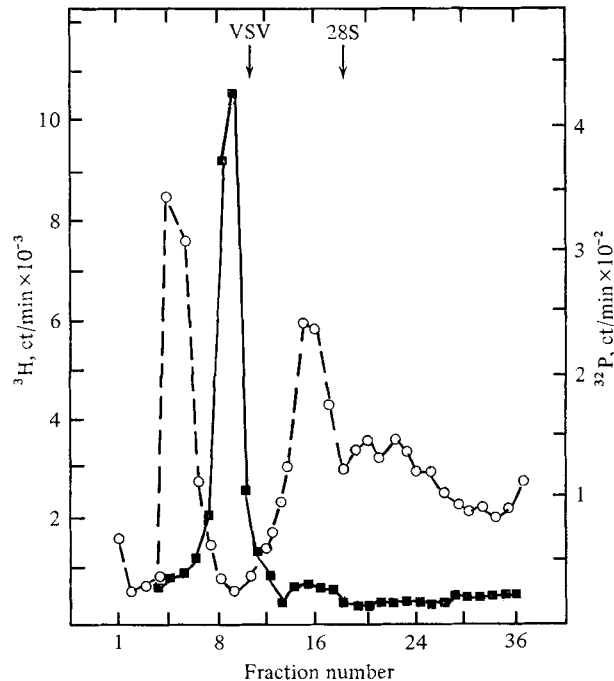


Fig. 3. Electrophoresis of JHM RNA and RSV RNA in 2% polyacrylamide-agarose gels. ^{32}P -RSV RNA was treated for 3 min at 80 °C and mixed with untreated RSV RNA (\circ - - \circ) and 28S-ribosomal RNA. ^3H -JHM RNA (\blacksquare — \blacksquare) was electrophoresed together in this gel and in combination with ^3H -VSV RNA and 28S-ribosomal RNA in parallel gels.

Table 1. *Binding of genomic RNA labelled with ^3H -uridine to oligo(dT)-cellulose columns*

RNA	Total ct/min	% bound
JHM batch 1	93 520	40
JHM batch 2	35 600	36
JHM batch 3	17 300	37
JHM batch 4	32 500	45
JHM batch 1 + poly (U)	81 400	< 2
28S RNA	23 000	< 2

Polyadenylate sequences in JHM virus RNA

Between 36 and 45% of four different batches of JHM RNA bound to oligo-(dT) cellulose (Table 1). Less than 2% of a similar amount of ^3H -28S ribosomal RNA bound under the same conditions. The specificity of the binding was further demonstrated by hybridizing the RNA to an excess of polyuridylic acid before chromatography on oligo-(dT) cellulose. We conclude from these data, that at least 35 to 40% of the virion RNA is polyadenylated.

In one experiment the integrity of RNA recovered from bound and unbound fractions was checked by velocity sedimentation. Both bound and unbound RNA migrated as 52 to 54S peaks which were broader than that of the starting material before chromatography. It may be, therefore, that some degradation occurred during the binding procedure on oligo-(dT) cellulose columns.

To establish further the presence of poly A sequences in at least a portion of JHM virus RNA, purified RNA labelled with either ^3H -adenosine or ^3H -uridine was digested in parallel

Table 2. Relative sensitivities of JHM RNA against RNase*

RNA	% resistance		adenosine-uridine
	³ H-adenosine	³ H-uridine	
JHM batch 1	0.66 %	0.16 %	+0.50 %
JHM batch 2	0.78 %	0.24 %	+0.54 %
JHM batch 3	0.97 %	0.20 %	+0.77 %

* Conditions of treatment: RNase A 20 μ g, RNase T1 450 units/200 μ l virus RNA 5 (10 μ g), yeast RNA 25 μ g (30 min, 37 °C).

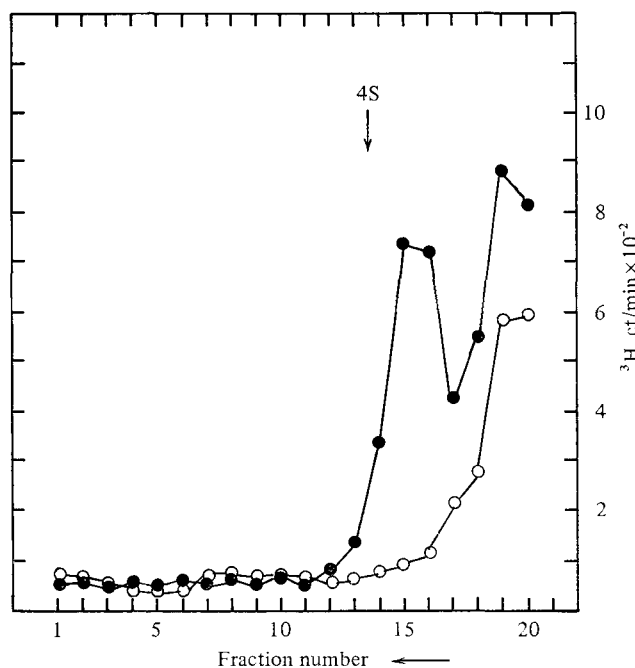


Fig. 4. Velocity sedimentation of JHM RNA after digestion with ribonuclease A and ribonuclease T1. Purified JHM RNAs labelled with ³H-adenosine (●—●) or ³H-uridine (○—○) were digested and centrifuged in parallel gradients of 10 ml 5 to 20 % (w/w), sucrose with 0.5 % SDS for 12 h at 40000 rev/min. *Escherichia coli* tRNA was added as marker (indicated by the arrow labelled 4S). Radioactivity was measured after trichloroacetic acid precipitation.

incubations with a mixture of ribonuclease A and ribonuclease T1. In all cases (Table 2) the percentage of nucleotide sequences resistant to ribonuclease digestion as quantified by insolubility in trichloroacetic acid differed in the range of 0.6 % (± 0.15 %) between ³H-adenosine and ³H-uridine labelled RNA. This suggests a polyadenylate sequence in virus RNA of between 60 and 120 residues. This suggestion is further strengthened by sedimentation analysis of ribonuclease digested virion RNA labelled with ³H-uridine and ³H-adenosine (Fig. 4). RNA labelled with ³H-adenosine shows clearly a peak of material sedimenting at 2 to 4S resistant to ribonuclease digestion. No such material can be found with ³H-uridine labelled RNA.

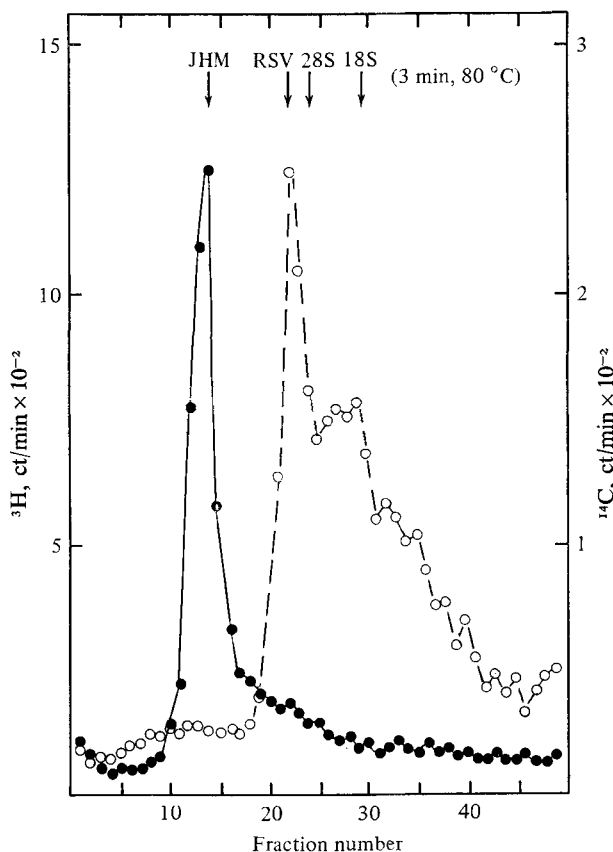


Fig. 5. Heat treatment of JHM RNA and RSV RNA. Purified ^3H -JHM RNA (●—●) and ^{14}C -RSV RNA (○—○) were heated for 3 min at 80°C in NET buffer and electrophoresed together with ribosomal marker RNA on the same gel (1.6% polyacrylamide-agarose). Untreated JHM RNA and RSV RNA were electrophoresed in a parallel gel as shown in Fig. 2. Treatment for 3 min at 100°C or with DMSO gave the same results.

Table 3. Infectivity of purified RNA from JHM virus for *Sac*(-) cells (cluster-plates with 2×10^5 cells/well)

RNA batch 1	Infected/total	RNA batch 2	Infected/total
$\mu\text{g/well}$		$\mu\text{g/well}$	
0.5	3/3	0.7	2/3
0.05	1/3	0.07	0/3
0.005	0/3	0.007	0/3
0.5		0.7	
+ ribonuclease A, 5 μg 15 min 37°C before adsorption	0/3	+ ribonuclease A, 5 μg 15 min 37°C before adsorption	0/3

Integrity and infectivity of JHM virus RNA

Purified RNA was dissolved in NET buffer and heated at 80 or 100°C for 3 min and quenched in ice. The treated RNA was analysed either in velocity gradients or by agarose-polyacrylamide gel electrophoresis (Fig. 5). The electrophoretic behaviour of heat treated JHM virus RNA does not differ from the untreated material. These findings indicate that the genomic RNA of JHM virus is a continuous strand. That the conditions were sufficient to

dissociate non covalently linked RNA to subunits was shown by the melting of RSV RNA. Further evidence was obtained by melting with DMSO. Similar to the picture obtained after heat treatment no change in the migration of JHM virus RNA was observed.

If the genomic RNA of JHM virus is functioning as a polyadenylated messenger RNA, it should be infectious. Two batches of purified RNA, one prepared by lysis with SDS and one by phenol extraction before purification, were tested for infectivity on Sac(-) cells (Table 3). C.p.e. occurred within three days. The isolated virus was identified by neutralization with anti JHM serum. The RNA concentration used was determined by monitoring the absorbance at 260 nm of a sample from the concentrated RNA. A gross calculation from the data in Table 3 shows that 1 μ g of RNA corresponds to a range of 3 to 10 p.f.u. of virus.

DISCUSSION

The data presented indicate that the genome of the murine coronavirus JHM consists of a single-stranded, infectious RNA containing polyadenylated sequences. The apparent mol. wt. is between 5.4 and 6.5×10^6 . No evidence for dissociation to subunits under conditions sufficient to denature RSV RNA was found.

Our estimation of the mol. wt. is based on velocity sedimentation in sucrose gradients and agarose-polyacrylamide gel electrophoresis in comparison to the marker RNAs of VSV, RSV and ribosomes. The values obtained by velocity sedimentation and electrophoresis in 1.6 and 2% polyacrylamide-agarose gels were consistent and correlated with the mol. wt. of 5.5 to 5.7×10^6 reported for avian bronchitis virus by Schochetman *et al.* (1977) and 6.1×10^6 for the human strain OC 43 (Tannock & Hierholzer, 1977) determined by similar techniques. The electrophoresis of a single-stranded RNA of high mol. wt. in polyacrylamide-agarose gels can only give approximate estimations because the secondary structure of the RNA influences the migration relative to the markers (Loening, 1969). Methods which are independent of hydrodynamic properties like length measurements by electron microscopy (Delius & Westphal, 1973) or ribonuclease T1-resistant oligonucleotide analysis may result in a more accurate figure of the actual mol. wt.

Different results were reported for the mol. wt. of avian bronchitis virus RNA by several groups. A mol. wt. in the range of 8 to 9×10^6 was either obtained by polyacrylamide gel electrophoresis (Watkins *et al.* 1975; MacNaughton & Madge, 1977) or by applying methods such as electrophoresis in agarose gels containing the chaotropic agent methylmercuric hydroxide, velocity sedimentation in formamide gradients and ribonuclease T1-resistant oligonucleotide analysis (Lomniczi & Kennedy, 1977). The latter results suggest that the actual size of the genomic RNA might not be exactly the same for all members of the coronavirus group.

The results obtained by chromatography on oligo-(dT) cellulose columns reveal that at least 36 to 45% of the JHM virus RNA is polyadenylated. A degree of polyadenylation similar to our findings was recently demonstrated for the mouse hepatitis strain MHV 2 (Yogo *et al.* 1977) and avian bronchitis virus (Lomniczi, 1977; MacNaughton & Madge, 1977; Schochetman *et al.* 1977). However, from these data the exact proportion of the genomic RNA molecules which are polyadenylated cannot be decided. It is conceivable that secondary structure of the comparatively big RNA molecules and poly(A) sequences of very small size inevitably reduces the actual amount bound to oligo(dT)-cellulose.

The existence of polyadenylation together with the infectivity of purified genomic RNA in both murine and avian strains suggests that the genomic RNA of coronaviruses is of messenger polarity.

Therefore, the Coronaviridae should be defined as a group of positive stranded animal viruses (class IV) by the definition of Baltimore (1971). This class comprises picornaviruses, togaviruses and several plant viruses. Polyadenylation need not be an inevitable feature of all coronaviruses. It is known that within picornaviruses different degrees or even lack of poly(A) sequences are found, in spite of the fact that the genomes of these viruses are infectious and can be translated *in vitro* (Frisby *et al.* 1976).

Both our results and the reports on avian bronchitis viruses support the conclusion that the coronavirus genome is a single-stranded, continuous RNA. This stands in obvious contrast to the results obtained from the porcine strains of transmissible gastroenteritis virus and haemagglutinating encephalitis virus (Garwes *et al.* 1975). In those studies heating of virus lysed with SDS led to a change of the mol. wt. in a manner similar to that found for oncornaviruses. No evidence for such a subunit structure was obtained for RNA of JHM virus by using similar conditions as described by Duesberg (1968). The observed subunit structure in porcine coronaviruses could be due to a contamination by RNA tumour viruses. We found that JHM virus cultured in L929 cells induced the production of endogenous type C particles. Cells derived from porcine tissue are also known to contain the genome of type C viruses (Lieber *et al.* 1975).

The results presented here for the murine strain JHM and the recent reports on other strains demonstrate that the genome of these viruses consists of a continuous RNA which is infectious and, therefore, of messenger polarity. This essential feature might be true for all coronaviruses and should prove useful in defining the taxonomic status and further experimental work on the coronavirus group.

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