J. gen. Virol. (1978), **39**, 497–504
Printed in Great Britain

The Genome of Human Coronavirus Strain 229E

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(*Accepted* 23 *December* 1977)

SUMMARY

The genomic RNA of human coronavirus strain 229E (HCV 229E) migrated on polyacrylamide gels as a single peak with a mol. wt. of $5{\cdot}8 \times 10^6$. Denaturation of the genome with formaldehyde did not alter its electrophoretic mobility, which suggests that the HCV 229E genome is a single-stranded molecule. At least 30 % of the genomic RNA was shown to contain covalently attached polyadenylic acid [poly(A)] sequences by binding the RNA to an oligo(dT)-cellulose column. These poly(A) tracts were shown to be about 70 nucleotides in length by measuring the resistance to digestion of HCV 229E RNA with pancreatic and T_1 RNases. Finally, the genomic RNA was shown to terminate at or near the 3′-terminus on the basis of its susceptibility to polynucleotide phosphorylase.

INTRODUCTION

Human coronavirus strain 229E (HCV 229E) is classified as a member of the coronaviruses (Tyrrell *et al.* 1968). The coronaviruses are grouped together primarily on their similar morphology in that they are all lipid-containing enveloped viruses ranging in diam. from 80 to 120 nm and possess widely-spaced club-shaped surface projections up to 20 nm in length (McIntosh, 1974). The properties of the genomes of certain coronaviruses have been reported. Avian infectious bronchitis virus (IBV) (Watkins *et al.* 1975; Lomniczi, 1977; Macnaughton & Madge, 1977; Schochetman *et al.* 1977), transmissible gastroenteritis virus (TGEV) (Garwes *et al.* 1975) and HCV strain OC43 (Tannock & Hierholzer, 1977) have been shown to consist of a large mol. wt. single single-stranded RNA molecule. Furthermore, the genome of IBV has been shown to contain covalently attached polyadenylic acid [poly(A)] sequences (Lomniczi, 1977; Macnaughton & Madge, 1977; Schochetman *et al.* 1977).

In this paper we have shown that the HCV 229E genome also exists as a single single-stranded RNA molecule of mol. wt. about $5{\cdot}8 \times 10^6$ with at least 30 % of the molecules containing poly(A) sequences of about 70 nucleotides in length, located at or near the 3′-terminus of the molecule.

METHODS

Virus culture. HCV strain 229E was obtained from Dr S. Reed, MRC Common Cold Unit, Salisbury and grown in monolayer cultures of diploid human embryo lung cells of the MRC continuous (MRCc) line. The cell monolayers were infected at an imput multiplicity of 0·1 infectious particles per cell and, following an adsorption period of 1 h at 33 °C, were incubated at 33 °C for 32 h in Eagle's BME with 2 % new born calf serum.

Preparation of labelled virus. Monolayers of cells were infected as above and 6 h post

infection 20 μCi/ml of ^{3}H-uridine or ^{3}H-adenosine (25 mCi/mmol) (Radiochemical Centre, Amersham) were added to the medium. In other experiments, 20 μCi/ml of ^{32}P-orthophosphate (88 to 140 Ci/mg phosphorus) (Radiochemical Centre, Amersham) were added to monolayers of cells in Eagle's BME with 2 % new born calf serum but without phosphate, immediately after the adsorption period. In all cases after incubation for 32 h at 33 °C, the cells were harvested and subjected to three freeze-thaw cycles.

Virus purification. All the purification steps were performed at 0 to 4 °C. The virus suspension was clarified at 2000*g* for 30 min at 4 °C and then the virus was pelleted at 75000*g* for 1 h. The pellet was resuspended in 1 ml of Dulbecco's phosphate buffered saline 'A' (PBSA), overlaid on to a linear 25 to 55 % (w/w) sucrose gradient in PBSA and centrifuged at 90000*g* for 16 h. One ml fractions were collected from these gradients and samples were measured for acid-precipitable radioactivity in a Packard Tri-Carb scintillation counter. The virus peak fractions were collected, and where necessary diluted again in PBSA and layered on to another linear 25 to 55 % (w/w) sucrose gradient in PBSA and centrifuged at 90000*g* for 16 h. Again, 1 ml fractions were collected and their acid-precipitable radioactivity determined. The peak fractions were used for further study.

Electron microscopy. Virus samples were examined after negative staining with 2 % (w/v) potassium phosphotungstate, pH 6·5, in a Philips EM 300 electron microscope.

Extraction of RNA from purified virus and MRCc cells. Virus particles from pooled fractions of sucrose gradients were pelleted at 75000*g* for 1 h, then extracted using proteinase K as previously described (Macnaughton *et al.* 1974). The virus was suspended in 15 mM-KCl, 10 mM-tris, pH 7·5, containing 1·5 % sodium dodecyl sulphate (SDS) and proteinase K (40 μg/mg virus; Merck) for 10 min at 0 °C, then incubated for 30 min at 25 °C. The solution was adjusted to 0·4 M in NaCl and 1 mM in EDTA, then 3 vol. of ethanol were added and stored at 4 °C for 16 h. The RNA was recovered by centrifuging at 1500*g* for 15 min. Unlabelled rRNA was extracted from MRCc cells as described previously (Macnaughton *et al.* 1974).

Polyacrylamide gel electrophoresis. Ten cm polyacrylamide gels (2·2 %), supported by 0·5 % agarose, were made by the procedure of Loening (1967) with certain modifications (Macnaughton *et al.* 1976). After a pre-electrophoresis of 30 min at 50 V, RNA was loaded on to each gel and subjected to electrophoresis for 2 h at 50 V. After electrophoresis, the gels were extruded, frozen and sliced into 1 mm discs. The gel slices were dissolved in hydrogen peroxide at 80 °C for 2 h; scintillation fluid was then added and radioactivity was determined in a Packard Tri-Carb scintillation counter.

Oligo(dT)-cellulose chromatography. Poly(A)-containing RNA molecules were separated from RNAs with no poly(A) sequences by binding them to 20 × 5 mm oligo(dT)-cellulose columns (Aviv & Leder, 1972). Polyadenylated RNA bound to columns in a high salt buffer (0·4 M-NaCl, 0·01 M-tris, pH 7·6, 1 mM-EDTA, 0·1 % SDS) and was eluted from the columns in a low salt buffer (0·01 M-tris, pH 7·6, 1 mM-EDTA, 0·1 % SDS). Samples of the fractions binding and not binding were assayed for acid-precipitable radioactivity by counting in a Packard Tri-Carb scintillation counter. Poly(A) and poly(U) were obtained from Sigma London Chemical Company Limited.

Nuclease digestions. To estimate the proportion of poly(A) in the HCV 229E genome and hence the size of the poly(A) tract, HCV 229E RNA was digested at 37 °C for 30 min in a solution containing 200 mM-NaCl, 20 mM-tris-HCl (pH 7·5), 10 μg/ml pancreatic ribonuclease A and 30 units/ml T_1 ribonuclease. Under these digestion conditions the poly(A) sequence is not destroyed. Digestion was terminated by the addition of SDS to a concentration of 1 %.

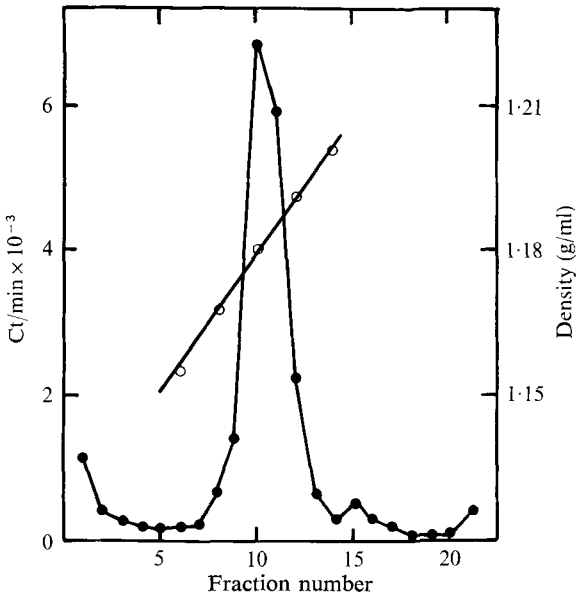

Fig. 1. Centrifugation of partially purified HCV 229E labelled with ^{3}H-uridine on 25 to 55 % (w/w) sucrose density gradients for 16 h at 90000*g*. ○——○, Distribution of density; ●——●, distribution of radioactivity.

Controlled digestion with the enzyme polynucleotide phosphorylase is known to phosphorylyse RNA in a 3′ to 5′ direction. Phosphorylosis was carried out at 37 °C in a solution containing 500 μg/ml of polynucleotide phosphorylase in 0·1 M-tris-HCl, pH 8·5, 5 mM-$MgCl_2$, 10 mM-sodium phosphate. The reaction was terminated by the addition of SDS to a concentration of 1 %.

RESULTS

Purification of HCV 229E

Labelled HCV 229E virus was centrifuged to equilibrium in a linear 25 to 55 % (w/w) sucrose density gradient (Fig. 1). A single peak of radioactivity was observed at a density of 1·18 g/ml, which was coincident with a peak of infectivity.

Virus particles with typical coronavirus morphology were observed in the peak fractions 10 and 11 (Fig. 2). The particles had complete coronas of surface projections and their membranes remained intact, as judged by the exclusion of stain from the centre of the particles.

Polyacrylamide gel electrophoresis of HCV 229E RNA

Analysis of HCV 229E RNA extracted by the standard phenol method (Kirby, 1965) produced a range of RNA species in the 50 to 15S region of sucrose gradients, similar to results described for other coronaviruses using similar extraction procedures (Tannock, 1973; Watkins *et al.* 1975; Tannock & Hierholzer, 1977). However, extraction of ^{3}H-uridine labelled HCV 229E RNA with proteinase K (Macnaughton *et al.* 1974) revealed a single peak of radioactivity at about 52S on 25 to 55 % sucrose gradients, using MRCc rRNA as marker.

In order to determine the mol. wt. of this RNA, ^{3}H-uridine labelled RNA was resolved on 2·2 % polyacrylamide gels and a single peak of activity was obtained (Fig. 3*a*). The mol. wt. of this RNA was estimated as $5{\cdot}8 \times 10^6$, by reference to cellular rRNAs (mol. wt. of

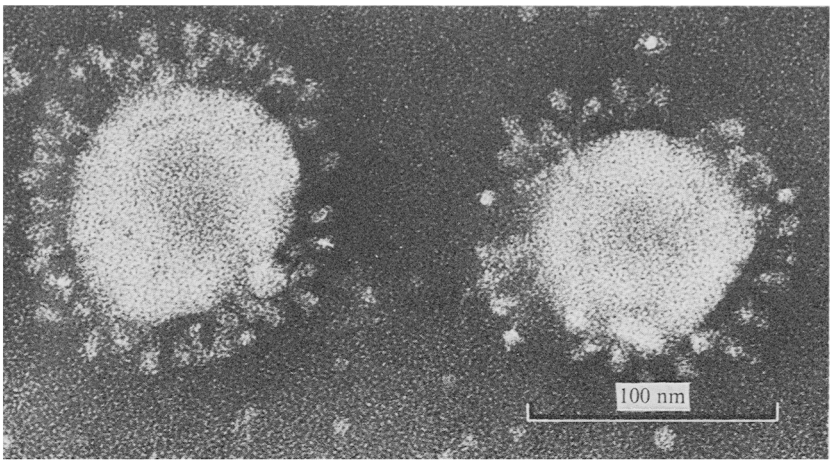

Fig. 2. HCV 229E virus particles of density 1·18 g/ml from sucrose density gradients. Negative staining with 2 % potassium phosphotungstate, pH 6·5.

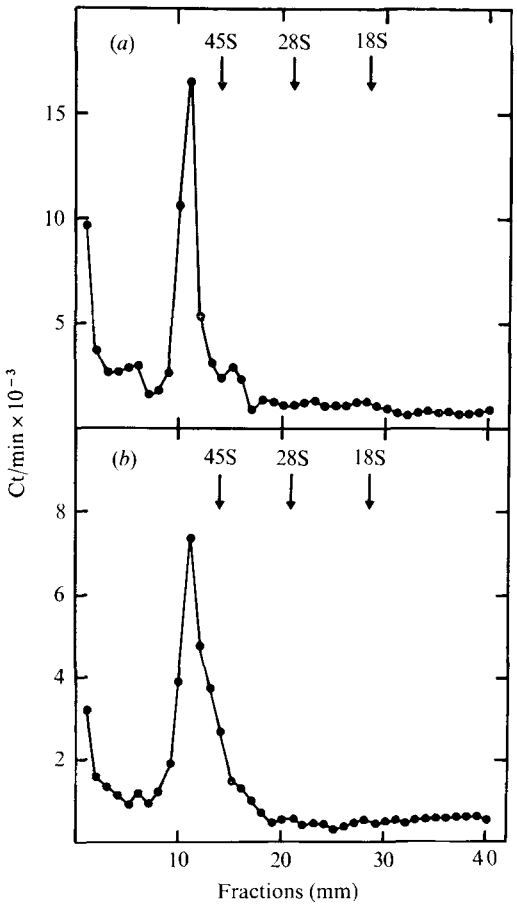

Fig. 3. Electrophoresis on a 2·2 % polysacrylamide gel of HCV 229E RNA labelled with ^{3}H-uridine. (*a*) Untreated RNA, (*b*) RNA treated with 10 % formaldehyde for 20 min at 67 °C. The arrows indicate the positions of untreated MRCc rRNAs which were co-electrophoresed on the same gels.

$1{\cdot}64 \times 10^6$ and $0{\cdot}67 \times 10^6$, Petermann & Pavlovec, 1966) and their 45S precursor ($4{\cdot}1 \times 10^6$; Weinberg & Penman, 1970).

^{3}H-uridine labelled HCV 229E RNA, denatured by heating with 10 % formaldehyde for 20 min at 67 °C (Macnaughton *et al.* 1974), was run on 2·2 % polyacrylamide gels (Fig. 3*b*). The mol. wt. of denaturated HCV 229E RNA was essentially the same as that of untreated RNA. This result shows that the HCV 229E genome is not composed of multiple subunits and contains little, if any, double strandedness.

Table 1. *Binding of various RNAs to oligo(dT)-cellulose columns*

Labelled RNA species	Initial ct/min	Binding to oligo(dT)-cellulose ct/min	Binding to oligo(dT)-cellulose %
Poly(A)	53014	52997	100
MRCc rRNA	13872	278	2
Poly(U)	42635	109	0
HCV 229E RNA*	27690	8842	32
	19932	5601	28
	12625	3331	26

* Three different HCV 229E RNA preparations were used and the data shown for each preparation represent averages of triplicate determinations.

Binding to oligo(dT)-cellulose

The data presented in Table 1 shows that between 25 and 30 % of HCV 229E RNA bound to oligo(dT)-cellulose columns. Under the same conditions of oligo(dT)-cellulose chromatography essentially no RNA species lacking poly(A) tracts [i.e. MRCc rRNA and poly(U)] bound to the columns, while poly(A) bound 100 %. These results indicate that about 30 % of the HCV 229E RNA contains a covalently bound poly(A) sequence.

Digestion with pancreatic ribonuclease A and T_1 ribonuclease

Labelled HCV 229E RNA that had bound to an oligo(dT)-cellulose column was digested with a mixture of pancreatic ribonuclease A (10 μg/ml) and T_1 ribonuclease (30 units/ml) for 30 min at 37 °C in order to obtain the poly(A) portion of the HCV 229E genome, which

Table 2. *Binding of labelled HCV 229E RNA to oligo(dT)-cellulose columns after digestion with nucleases**

Labelled HCV 229E RNA	Initial ct/min	Binding to oligo(dT)-cellulose ct/min	Binding to oligo(dT)-cellulose %
^{32}P-orthophosphate	22168	87	0·4
labelled	24734	96	0·4
^{3}H-adenosine	29014	434	1·5
labelled	28096	453	1·6
^{3}H-uridine	26275	24	0·1
labelled	27572	24	0·1

* Digestion was at 37 °C for 30 min in a solution containing 200 mM-NaCl, 20 mM-tris-HCl (pH 7·5). 10 μg/ml pancreatic ribonuclease A and 30 units/ml T_1 ribonuclease.

is not destroyed under these conditions. Table 2 shows the results of such an experiment using a number of differently labelled RNA preparations. About 0·4 % of the HCV 229E genome remained from ^{32}P-orthophosphate labelled preparations, indicating that 0·4 % of the HCV 229E genome consists of poly(A). As the ribonuclease resistant radioactivity was

Table 3. *Removal of poly(A) from HCV 229E RNA with polynucleotide phosphorylase**

Length of incubation with polynucleotide phosphorylase	Initial ct/min	Binding to oligo(dT)-cellulose	
		ct/min	%
Control a†	17534	4784	27
Control b‡	22014	6428	29
2 min	27670	4685	17
	31726	6226	20
10 min	14198	1038	7
	16471	1669	10

* Phosphorylasis was carried out at 37 °C in a solution containing 500 μg/ml polynucleotide phosphorylase in 0·1 M-tris-HCl (pH 8·5), 5 mM-$MgCl_2$, 10 mM-sodium phosphate.

† Sample incubated for 10 min without enzyme.

‡ Sample incubated for 10 min without 10 mM-sodium phosphate.

very low, ^{3}H-adenosine labelled HCV 229E RNA was digested under the same conditions in order to produce a higher and hence more accurate determination of ribonuclease resistant radioactivity. In this case about 1·6 % of the molecules were resistant to digestion. Assuming that approx. 1 in every 4 nucleotides is adenosine, then, again, an estimated 0·4 % of the genome consists of poly(A). Essentially, no ^{3}H-uridine labelled HCV 229E RNA showed any ribonuclease resistant radioactivity, indicating that the nuclease treatment had eliminated all the RNA sequences except the poly(A) tracts.

Polynucleotide phosphorylase digestion of HCV 229E RNA

Controlled digestion with the enzyme polynucleotide phosphorylase has been shown to remove 3′-terminal poly(A) from mRNA (Sheldon *et al.* 1972). This procedure was used to determine whether the poly(A) sequences of HCV 229E RNA were located at the 3′-terminus. Samples of HCV 229E RNA were digested with polynucleotide phosphorylase for 2 min and 10 min and the loss of poly(A) was monitored by the inability of RNA lacking poly(A) to bind to oligo(dT)-cellulose. Table 3 shows that digestion with polynucleotide phosphorylase for 10 min significantly reduced the fraction of HCV 229E RNA binding to oligo(dT)-cellulose from about 30 % (control a) to under 10 %.

Under the same conditions of digestion with polynucleotide phosphorylase, about 95 and 75 % of HCV 229E RNA remained undigested after 2 and 10 min respectively. This shows that, while only about a third of HCV 229E RNA molecules that had poly(A) sequences and had bound to oligo(dT)-cellulose, could do so after 10 min digestion with the enzyme, three quarters of HCV 229E RNA molecules remained intact after digestion under the same conditions. Thus there is a more rapid loss of poly(A) than RNA during phosphorylasis.

For these experiments to be valid it is essential that the polynucleotide phosphorylase contained no nuclease contaminants. Incubation of the HCV 229E RNA with polynucleotide phosphorylase but without phosphate (control b), produced no decrease in binding of the HCV 229E RNA to oligo(dT)-cellulose compared with control a, which contained no polynucleotide phosphorylase. Thus, the polynucleotide phosphorylase preparation did not contain any major nuclease contaminants. These results are interpreted to mean that the HCV 229E RNA has 3′-terminal poly(A) sequence(s).

DISCUSSION

We have shown that the RNA genome of HCV 229E migrated as a single high mol. wt. species on 2·2 % polyacrylamide gels. Denaturation with 10 % formaldehyde did not change the mobility of this RNA species. These results support the conclusion that the genome of HCV 229E consists of a large mol. wt. single single-stranded RNA species. Similar observations have been made with the coronaviruses: IBV (Watkins *et al.* 1975; Lomniczi, 1977; Macnaughton & Madge, 1977; Schochetman *et al.* 1977), TGEV (Garwes *et al.* 1975) and HCV OC43 (Tannock & Hierholzer, 1977).

A number of reports show that the coronavirus genome is fragile. Tannock (1973) and Watkins *et al.* (1975), using IBV, and Tannock & Hierholzer (1977), using HCV OC43, have shown that coronavirus RNA extracted with phenol was very heterogeneous. Furthermore, Garwes *et al.* (1975) and Tannock & Hierholzer (1977) observed that heating TGEV and HCV OC43 RNAs, respectively, dissociated the RNA molecules and material of about 4S accumulated. However, Watkins *et al.* (1975), Macnaughton & Madge (1977), Schochetman *et al.* (1977) and Lomniczi (1977) have observed no genome degradation on heating IBV RNA. Certainly, our results show that denaturation of HCV 229E RNA with 10 % formaldehyde at 67 °C for 20 min did not produce any dissociation of the molecules on polyacrylamide gels. Similarly, Schochetman *et al.* (1977) denatured the IBV genome by heating at 100 °C for 1 min but observed no change in the electrophoretic mobility of the RNA.

There is some disagreement on the mol. wt. of coronavirus RNAs – estimates vary from $9{\cdot}0 \times 10^6$ (Watkins *et al.* 1975) to $5{\cdot}6 \times 10^6$ (Schochetman *et al.* 1977). Our results suggest that the mol. wt. of HCV 229E RNA is in the lower range of these estimates at about $5{\cdot}8 \times 10^6$. The variations observed in the mol. wt. of coronavirus genomes are not unexpected as high mol. wt. RNAs are not readily resolved on low percentage polyacrylamide gels (Loening, 1967) and the mol. wt. of coronavirus RNAs are generally extrapolated beyond that of the largest marker.

The mol. wt. of the HCV 229E genome of $5{\cdot}8 \times 10^6$ corresponds to about 18000 nucleotides. We have shown that about 0·4 % of the genome of polyadenylated molecules consists of poly(A), which represents a poly(A) sequence of about 70 nucleotides. This sequence has been shown to occur at or near the 3′-terminus of the molecule.

Other reports have indicated that the IBV genome also contains poly(A) sequences (Lomniczi, 1977; Macnaughton & Madge, 1977; Schochetman *et al.* 1977) at or near the 3′-terminus (M. R. Macnaughton, unpublished results). We do not know at present whether the poly(A) tracts are located in a single segment or in separate smaller segments. However, by analogy with other polyadenylated virus genomes, it would be expected that the poly(A) exists as a single segment at the 3′-terminus of the genome. These results suggest that the genome of HCV 229E may have a messenger function. This hypothesis is strongly supported by the results showing that IBV RNA is infectious (Lomniczi, 1977; Schochetman *et al.* 1977) and that IBV particles do not exhibit an RNA-dependent RNA polymerase activity (Schochetman *et al.* 1977). We have not been able to show that HCV 229E RNA is infectious, although this is probably due to technical difficulties. However, no RNA-dependent RNA polymerase activity has been observed associated with virus particles (M. H. Madge, unpublished results). Further experiments are in progress in order to clarify the possible messenger function of the HCV 229E genome.

We thank Dr D. A. J. Tyrrell for his advice and criticism and Mrs Heather A. Davies for the electron microscopy.

REFERENCES

AVIV, H. & LEDER, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 1408–1412.

GARWES, D. J., POCOCK, D. H. & WIJASZKA, T. M. (1975). Identification of heat-dissociable RNA complexes in two porcine coronaviruses. *Nature, London* **257**, 508–510.

KIRBY, K. S. (1965). Isolation and characterization of ribosomal ribonucleic acid. *Biochemical Journal* **96**, 266–269.

LOENING, U. E. (1967). The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. *Biochemical Journal* **102**, 251–257.

LOMNICZI, B. (1977). Biological properties of avian coronavirus RNA. *Journal of General Virology* **36**, 531–533.

MACNAUGHTON, M. R., FREEMAN, K. B. & BISHOP, J. O. (1974). A precursor to hemoglobin mRNA in nuclei of immature duck red blood cells. *Cell* **1**, 117–125.

MACNAUGHTON, M. R., COOPER, J. A. & DIMMOCK, N. J. (1976). Rhinovirus multistranded RNA: dependence of the replicative form on the presence of actinomycin D. *Journal of Virology* **18**, 926–932.

MACNAUGHTON, M. R. & MADGE, M. H. (1977). The characterisation of the virion RNA of avian infectious bronchitis virus. *FEBS Letters* **77**, 311–313.

MCINTOSH, K. (1974). Coronaviruses: a comparative review. *Current Topics in Microbiology and Immunology* **63**, 85–129.

PETERMAN, M. L. & PAVLOVEC, A. (1966). The subunits and structural ribonucleic acids of Jensen sarcoma ribosomes. *Biochimica et Biophysica Acta* **114**, 264–276.

SCHOCHETMAN, G., STEVENS, R. H. & SIMPSON, R. W. (1977). Presence of infectious polyadenylated RNA in the coronavirus avian bronchitis virus. *Virology* **77**, 772–782.

SHELDON, R., KATES, J., KELLEY, D. E. & PERRY, R. P. (1972). Polyadenylic acid sequences on 3′-termini of vaccinia messenger ribonucleic acid and mammalian nuclear and messenger ribonucleic acid. *Biochemistry* **11**, 3829–3834.

TANNOCK, G. A. (1973). The nucleic acid of infectious bronchitis virus. *Archiv für die gesamte Virusforschung* **43**, 259–271.

TANNOCK, G. A. & HIERHOLZER, J. C. (1977). The RNA of human coronavirus OC-43. *Virology* **78**, 500–510.

TYRRELL, D. A. J., ALMEIDA, J. D., BERRY, D. M., CUNNINGHAM, C. H., HAMRE, D., HOFSTAD, M. S., MALLUCCI, L. & MCINTOSH, K. (1968). Coronaviruses. *Nature, London* **220**, 650.

WATKINS, H., REEVE, P. & ALEXANDER, D. J. (1975). The ribonucleic acid of infectious bronchitis virus. *Archives of Virology* **47**, 279–286.

WEINBERG, R. A. & PENMAN, S. (1970). Processing of 45 S nucleolar RNA. *Journal of Molecular Biology* **47**, 169–178.

(*Received* 22 *September* 1977)