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Coronavirus JHM: Characterization of Intracellular Viral RNA

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SUMMARY

After infection of Sac(−) cells with the murine coronavirus JHM the synthesis of seven major and two minor RNA species was induced. These RNAs were polyadenylated and single-stranded. Their mol. wt. were estimated by electrophoresis in agarose gels containing methylmercury hydroxide. The values for the major species were $6{\cdot}67 \times 10^6$ for RNA of genome size (RNA 1), $3{\cdot}42 \times 10^6$ for RNA 2, $2{\cdot}76 \times 10^6$ for RNA 3, $1{\cdot}35 \times 10^6$ for RNA 4, $1{\cdot}19 \times 10^6$ for RNA 5, $0{\cdot}93 \times 10^6$ for RNA 6 and $0{\cdot}62 \times 10^6$ for RNA 7. The minor species have a size of $4{\cdot}7 \times 10^6$ (RNA a) and $1{\cdot}5 \times 10^6$ (RNA b). The same number of species were found by electrophoresis after denaturation with glyoxal–dimethyl sulphoxide. No gross difference in number of RNAs and the amount of each species was found between total cytoplasmic RNA, polyadenylated cytoplasmic RNA and RNA extracted from pelleted polysomes.

Coronaviruses are important pathogens (Robb & Bond, 1979) which cause diseases of economic significance such as avian bronchitis and transmissible gastroenteritis in pigs. In man, infections of both the respiratory and gastrointestinal system are induced by coronaviruses and a possible association with demyelinating central nervous system disorders has been discussed (Burks *et al.*, 1980). In this connection, the murine coronavirus JHM has received attention because of its ability to induce demyelinating central nervous diseases in mice and rats (Weiner, 1973; Nagashima *et al.*, 1978, 1979). However, little is known about the replication of this virus group in lytic and persistent infections.

So far, it has been shown that the genome of JHM virus consists of a polyadenylated, single-stranded infectious RNA with a mol. wt. of at least $5{\cdot}4 \times 10^6$ (Lai & Stohlman, 1978; Wege *et al.*, 1978). JHM virions contain six major proteins with a mol. wt. between 23×10^3 and 170×10^3. Four of these proteins are glycosylated (Wege *et al.*, 1979). Within the cells, several polypeptides have been detected with are specific to virus-infected cells and their possible relationship to the virion proteins has been investigated (Siddell *et al.*, 1981; Bond *et al.*, 1979). Studies on the cell-free translation of RNA from JHM-infected cells gave the first experimental evidence for the existence of several subgenomic mRNA's (Siddell *et al.*, 1980). Subsequently, Stern & Kennedy (1980) using T_1 oligonucleotide fingerprinting demonstrated that in cells infected with the avian coronavirus, infectious bronchitis virus, a set of five subgenomic polyadenylated positive-stranded RNAs are synthesized, which are presumed to function as mRNAs.

In this communication we now describe the number and size of the major RNA species found in cells infected with JHM virus. This information is essential as a basis for the characterization of the mRNAs of coronaviruses and their translation products.

To study the rate of JHM virus-specific RNA synthesis under single cycle growth conditions, Sac(−) cells in suspension cultures were infected with an m.o.i. of 4 p.f.u./cell as described earlier (Siddell *et al.*, 1980) and pulsed at various times after infection for 1 h with ^{3}H-uridine in the presence of 1 μg/ml actinomycin D. This concentration of actinomycin D does not inhibit the synthesis of infectious virus under single cycle growth conditions, but rapidly stops the synthesis of host cell RNA. By determination of the trichloroacetic acid-precipitable radioactivity from cytoplasmic lysates, an increase in virus-specific RNA

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synthesis was first detected at about 4 h post-infection and the rate of RNA synthesis reached a peak at 9 h post-infection. The release of infectious virus paralleled the curve of intracellular viral RNA synthesis.

For preparation of cytoplasmic RNA, cells were labelled with ^{3}H-uridine in the presence of actinomycin D from 4 to 6·5 h post-infection, and the RNA was extracted by phenol–SDS from cell lysates prepared as described by Siddell *et al.* (1980). The extracted RNA was analysed by electrophoresis into 0·9% agarose gels after denaturation by either methylmercury hydroxide (Bailey & Davidson, 1976) or glyoxal–dimethyl sulphoxide (glyoxal–DMSO) (McMaster & Carmichael, 1977). Virion RNA labelled with ^{3}H-uridine was extracted from purified virus (Wege *et al.*, 1978) and electrophoresed after glyoxal–DMSO treatment. As the fluorograph in Fig. 1 (*a*, *b*) illustrates, the bulk of the virion RNA migrated as a single homogeneous band and considerably slower than vesicular stomatitis virus (VSV) RNA. The mol. wt. of JHM virion RNA, determined in relation to the markers indicated in Fig. 1 (*b*), was $6{\cdot}67 \times 10^6$ and is therefore bigger than estimated previously under non-denaturating conditions (Wege *et al.*, 1978). Electrophoresis of the total cytoplasmic RNA reproducibly revealed seven major and two minor species (Fig. 1 *c*). The nomenclature used here starts with the RNA of genome size as no. 1 and numbers the species in order of decreasing size, excepting species a and b which are minor components. The only species varying from batch to batch was the minor component designated RNA b.

Essentially identical results were seen after denaturation of the sample with methylmercury hydroxide or glyoxal–DMSO. The methylmercury hydroxide gel system was chosen for determinations of the mol. wt. of the RNA species (Fig. 1*d*). The mol. wt. were measured by co-electrophoresis of cytoplasmic RNA mixed with radioactively labelled marker RNAs consisting of *Escherichia coli* 16S and 23S RNA, ribosomal 18S and 28S RNA from L cells and virion RNA from VSV strain Indiana. The values for each individual RNA were obtained from four to six independent gels. The calibration curve from these determinations is shown in Fig. 2. The following values were found for cytoplasmic JHM RNA (mol. wt. $\times 10^{-6}$): RNA 1 (genome size), $6{\cdot}67 \pm 0{\cdot}46$; RNA a, $4{\cdot}7 \pm 0{\cdot}10$; RNA 2, $3{\cdot}42 \pm 0{\cdot}04$; RNA 3, $2{\cdot}76 \pm 0{\cdot}05$; RNA b, $1{\cdot}50 \pm 0{\cdot}09$; RNA 4, $1{\cdot}35 \pm 0{\cdot}04$; RNA 5, $1{\cdot}19 \pm 0{\cdot}04$; RNA 6, $0{\cdot}93 \pm 0{\cdot}02$; and RNA 7, $0{\cdot}62 \pm 0{\cdot}01$.

The number and relative amounts of subgenomic species were very similar in RNA extracted early (about 4 h post-infection) and late (about 8 h post-infection) in the infectious cycle. More than 50% of this cytoplasmic RNA binds to poly(U)-sepharose and is therefore polyadenylated. However, the number and relative quantity of each RNA species remained unchanged by poly(A) selection (Fig. 1 *e*). The species numbered 1 to 7 were sensitive to digestion by pancreatic RNase (data not shown). This was tested by electrophoresing ^{32}P-labelled cytoplasmic polyadenylated RNA in agarose–polyacrylamide–urea gels (Floyd *et al.*, 1974), cutting out the bands and re-extracting the RNAs with phenol–SDS and digestion with pancreatic RNase after purification of the RNA on formamide–sucrose gradients. Therefore, by the criterion of polyadenylation and RNase sensitivity most of the RNA species we describe are likely to be mRNAs and not replicative forms or intermediates.

To determine which RNA species are polysome-associated, cytoplasmic extracts obtained

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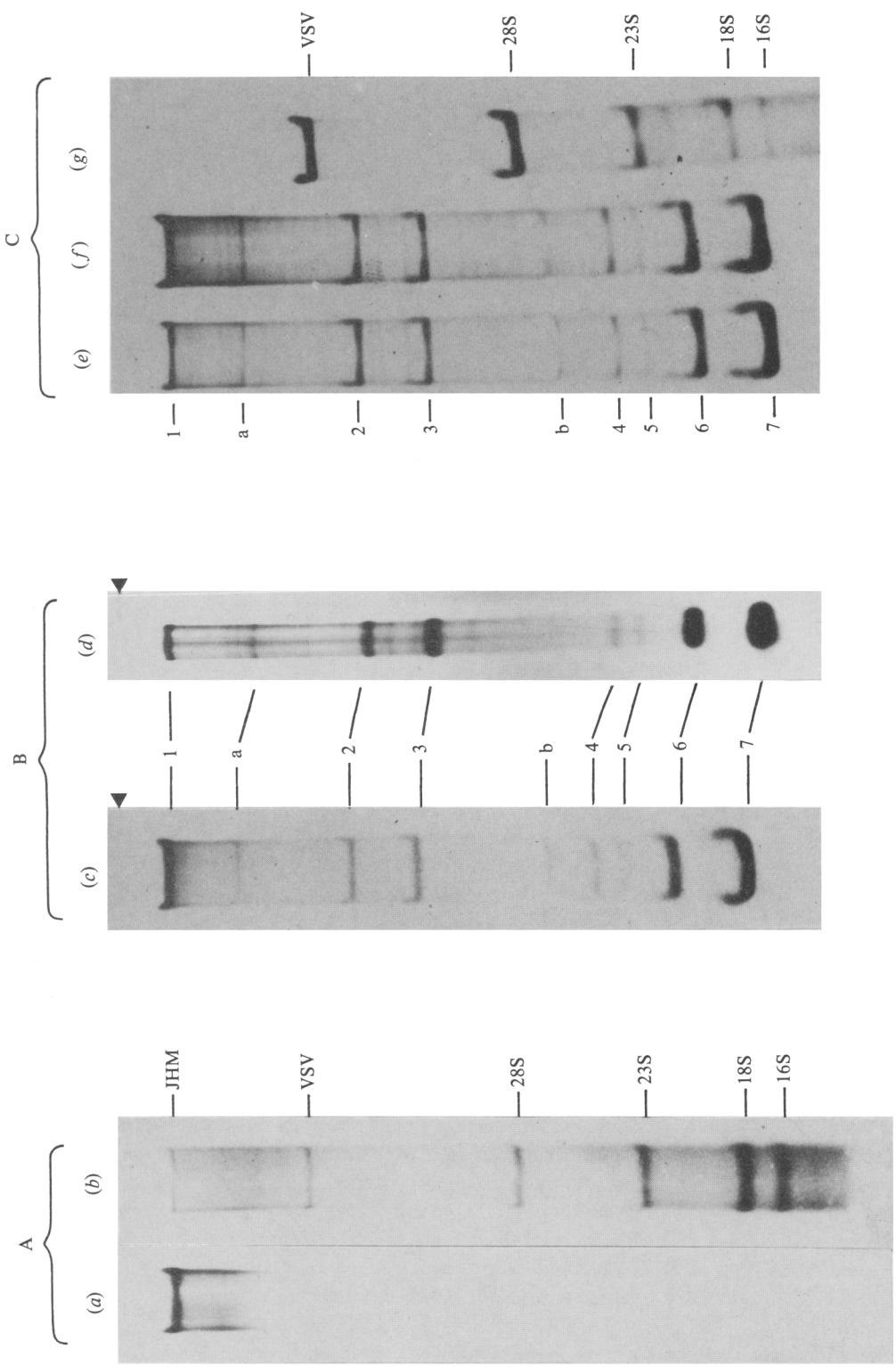
Fig. 1. Fluorograms of JHM RNAs separated by electrophoresis in 0·9% agarose gels. Denaturation of RNA samples was carried out with either glyoxal–DMSO or methylmercury hydroxide. Panel A, Virion RNA extracted from purified virus (*a*). The same sample was electrophoresed after mixing with marker RNAs (*b*). The mol. wt. of the marker RNAs are given in the legend to Fig. 2. Panel B, Total cytoplasmic RNA: (*c*) denaturation with glyoxal–DMSO and (*d*) denaturation with methylmercury hydroxide. Panel C, Cytoplasmic RNA selected for poly(A) (*e*) and RNA extracted from a polysomal pellet (*f*) prepared as described in the text. (*g*) Marker RNAs. The mol. wt. are included in the legend to Fig. 2.


A
(a)
(b)
JHM
VSV
28S
23S
18S
16S
B
(c)
(d)
1
a
2
3
b
4
5
6
7
C
(e)
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VSV
28S
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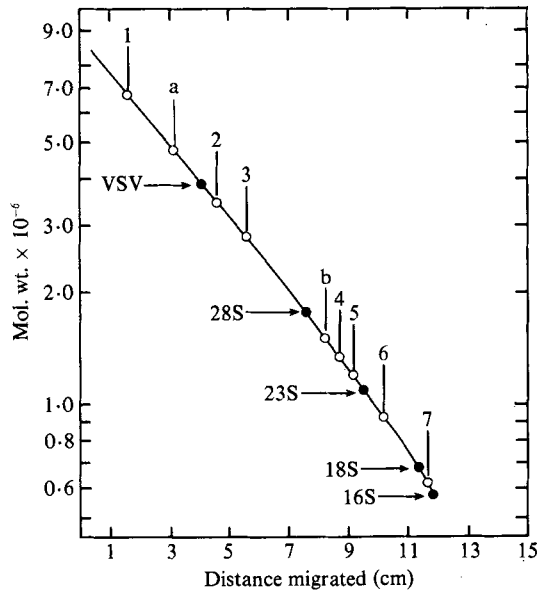

Fig. 2. Calibration curve for determination of mol. wt. obtained by electrophoresis of RNAs in 0·9% agarose–methylmercury hydroxide gels. The following marker RNAs were used: vesicular stomatitis virus genome (mol. wt. $3{\cdot}8 \times 10^6$; Repik & Bishop, 1971), ribosomal 18S and 28S RNA from L cells (mol. wt. $0{\cdot}68 \times 10^6$ and $1{\cdot}74 \times 10^6$; Loening, 1968) and *E. coli* 16S and 23S RNA ($0{\cdot}55 \times 10^6$ and $1{\cdot}07 \times 10^6$; Stanley & Bock, 1965). The positions of the individual intracellular RNAs relative to the marker RNAs are indicated by vertical bars.

by lysis with Nonidet P40 (NP40) were centrifuged through 15 to 40% sucrose gradients (Siddell *et al.*, 1980). The fractions sedimenting faster than the monosomal 80S peak were pooled and pelleted through a discontinuous gradient consisting of 2 M- and 0·5 M-sucrose. The RNA from the polysomal pellet was extracted by phenol–SDS and again selected for poly(A), denatured by glyoxal–DMSO treatment and electrophoresed in agarose. As can be seen by comparison of Fig. 1(*e*) with Fig. 1(*f*), no difference was found between RNA extracted from polysomes prepared as described above and poly(A)-containing cytoplasmic RNA. This strengthened our conclusion that most of the subgenomic species are mRNAs. If nucleocapsids are present in this polysomal preparation they could be expected in the region of polysomes sedimenting faster than 200S (Robb & Bond, 1979). By analysing the RNA extracted from polysomes fractionated on a sedimentation gradient, we indeed found a typical bimodal distribution of the radioactivity. RNA of genomic size is predominantly found in the region sedimenting faster than 200S (data not shown), but we could not demonstrate an EDTA-resistant peak in that region. This negative evidence obtained for JHM virus might be due to an extreme fragility of those structures relative to other murine coronaviruses.

In summary, six major polyadenylated single-stranded subgenomic RNA species were consistently demonstrable in addition to the genome-sized RNA during the replication of JHM virus in Sac(−) cells. The sizes of these RNAs were determined. By translation *in vitro* the smallest mRNA of JHM virus has been shown to code for a viral protein with a mol. wt. of 60×10^3 and the next smallest RNA codes for a viral protein with a mol. wt. of 23×10^3 (Siddell *et al.*, 1980). Both translation of RNA species eluted from agarose polyacrylamide–urea gels and electrophoresis of RNA size fractionated by sedimentation on formamide sucrose gradients confirmed the identity of RNA 7 and RNA 6 with the previously reported 17S and 19S mRNA (data not shown). Eucaryotic mRNAs are believed to initiate

translation only at the 5′-end, and these translation data suggest that only the cistron at the 5′-end of each JHM virus mRNA is translated. The size of the two smallest JHM virus mRNAs and their translation products are consistent with this concept.

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