CHARACTERISATION OF VIRAL RNA IN CELLS INFECTED WITH THE MURINE CORONAVIRUS JHM

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SUMMARY

The murine coronavirus JHM induces in Sac(-) cells seven major and two minor RNA species. These RNAs are polyadenylated and single stranded. Their sizes were estimated by electrophoresis in agarose gels containing methylmercury hydroxide. The mol. wts. for the major species are 6.67×10^6 for RNA of genome size, 3.42×10^6 for RNA 2, 2.76×10^6 for RNA 3, 1.35×10^6 for RNA 4, 1.19×10^6 for RNA 5, 0.93×10^6 for RNA 6 and 0.62×10^6 for RNA 7. The minor species have a size of 4.7×10^6 (RNA a) and 1.5×10^6 (RNA b). No essential difference in the number and proportion of each RNA species was found between total cytoplasmic RNA, polyadenylated cytoplasmic RNA and RNA extracted from pelleted polysomes, nor was any difference found during the infection cycle. The major RNA species are likely to be subgenomic mRNAs.

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INTRODUCTION

Mouse hepatitis viruses deserve special attention because of their potential for experimental studies of acute and chronic diseases. These agents induce a variety of diseases in small rodents (Robb et al. 1979). In tissue culture systems, both lytic and persistent infections are readily established (Lucas et al., 1978; Stohlman et al., 1979). The strain JHM is particularly interesting because of its ability to cause chronic disorders of the central nervous system in mice and rats (Weiner et al., 1973; Nagashima et al., 1979). However, little is known on the replication of this virus in lytic and persistent infections, a prerequisite for studies of the pathogenesis of these diseases.

The essential features of the JHM virus structure are now established. The genome of JHM virus consists of a single stranded polyadenylated RNA which is infectious and has a mol. wt. (in millions) of at least 5.6 (Lai and Stohlman, 1978; Wege et al., 1978). Purified virus consists of six major proteins with a mol. wt. between 23.000 and 170.000. Four of the proteins are glycosylated (Wege et al., 1979). In the infected cell, several virus specific polypeptides are synthesized and their possible relationship to the structural proteins have been investigated (Siddell et al., 1981; Bond et al., 1979). The cell free translation of RNA extracted from JHM infected cells provides strong evidence for the existence of several subgenomic mRNAs (Siddell et al., 1980). As a basis for the final characterisation of the mRNAs of JHM virus and their translation products we describe here the number and size of JHM virus specific RNA synthesized in infected cells.

MATERIAL AND METHODS

Details of virus growth, maintenance of cells, radioactive labelling, virus purification and extraction of RNA are described in previous communications (Wege et al. 1978 and 1979, Siddell et al., 1980).

Virus and cells. The JHM virus was originally obtained from L. Weiner, Johns Hopkins University, as a suckling mouse brain suspension. It was adapted to Sac (-) cells, a permanent murine Moloney sarcoma cell line (Weiland et al., 1978) and cloned by plaque passages.

Electrophoresis of RNA. RNA was elctrophoresed in 0.9 % agarose gels after denaturation with glyoxal-DMSO

(McMaster and Carmichael 1977). Alternatively, 0,9 % agarose gels containing 5 mM methylmercury hydroxide (Bailey & Davidson, 1976) were used. For fluorography, gels were soaked in Enhance (NEN-Chemicals), transferred into water, dried and exposed at - 70° C to Kodak XR5 film. RNA species were recovered from composite agarose polyacrylamide urea gels (Floyd et al., 1974) by excision, homogenisation and reextraction with phenol SDS. The extracted RNAs were poly A selected on poly U sepharose columns.

RESULTS

Kinetics of viral growth and RNA synthesis.

Suspension cultures of Sac (-) cells were infected with a m.o.i. of 4 p.f.u. per cell and pulsed at various

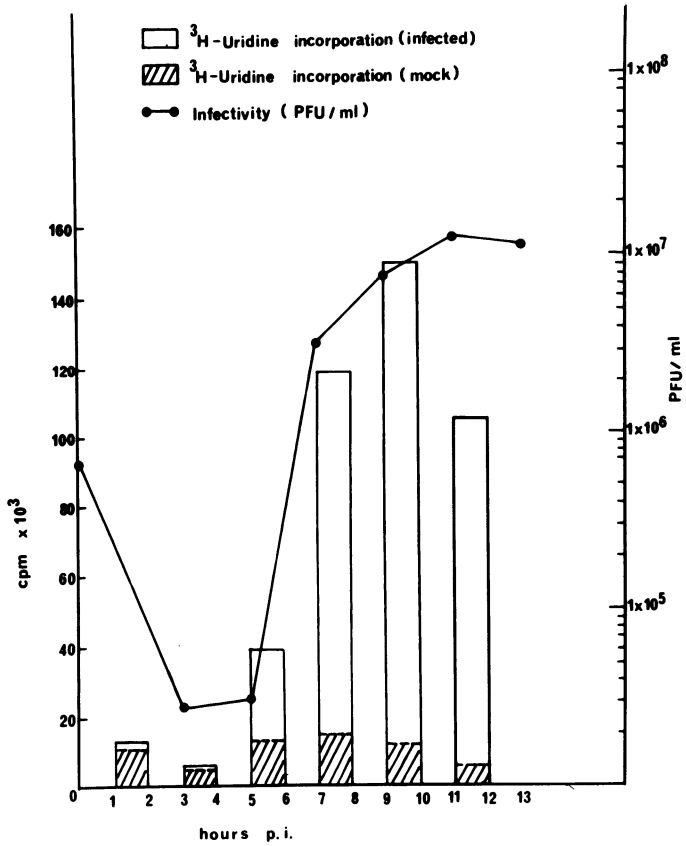


Fig.1: Rate of RNA synthesis and release of infectious virus from JHM infected Sac (-) cells (m.o.i. 4 p.f.u/ cell).

times for 1 h. Actinomycin D (1 µg/ml) was added before labelling with 3 H-uridine. At this concentration actinomycin D does not inhibit the growth of infectious virus. The rate of virus specific RNA synthesis was measured by determination of trichloracetic acid precipitable radioactivity from cytoplasmic lysates.

As Fig. 1 shows, an increase of virus specific RNA synthesis was first detected at about 4 h p.i. and the rate of RNA synthesis reached a peak at 9 h p.i.. Infectious virus was released into the culture medium in parallel to the production of RNA.

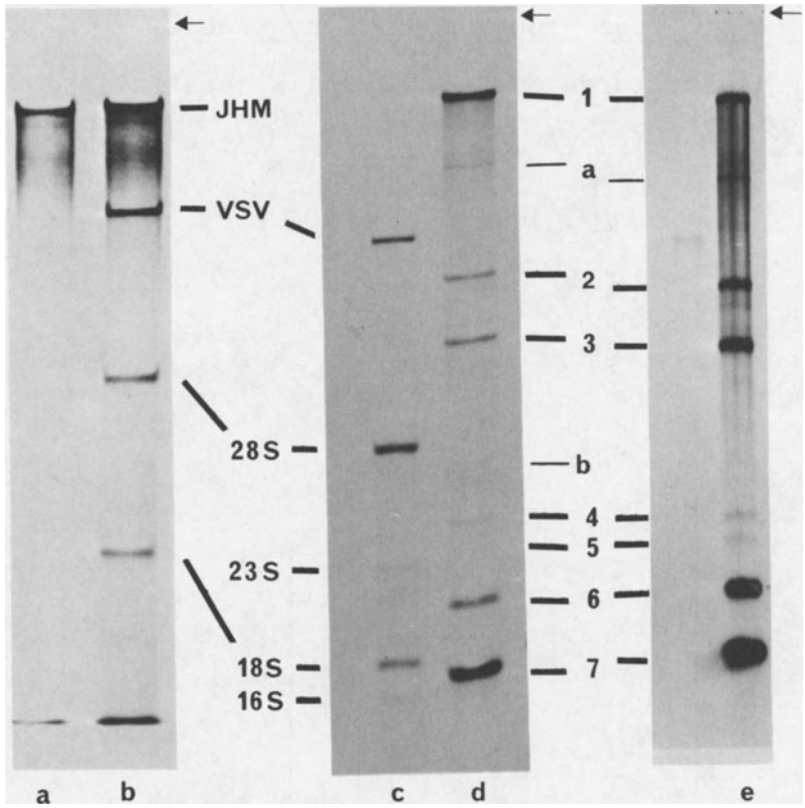


Fig. 2 Fluorograms of JHM-RNAs separated by electrophoresis in 0.9 % agarose gels. Denaturation of RNA samples either with glyoxal (slot a, b, c and d) or methylmercury hydroxide (slot e). a) and b) JHM virion RNA, c) marker-RNAs. Ribosomal RNA of E. coli and L-cells, VSV virion RNA, d) total cytoplasmic RNA denatured with glyoxal DMSO, e) total cytoplasmic RNA denatured with methylmercury hydroxide.

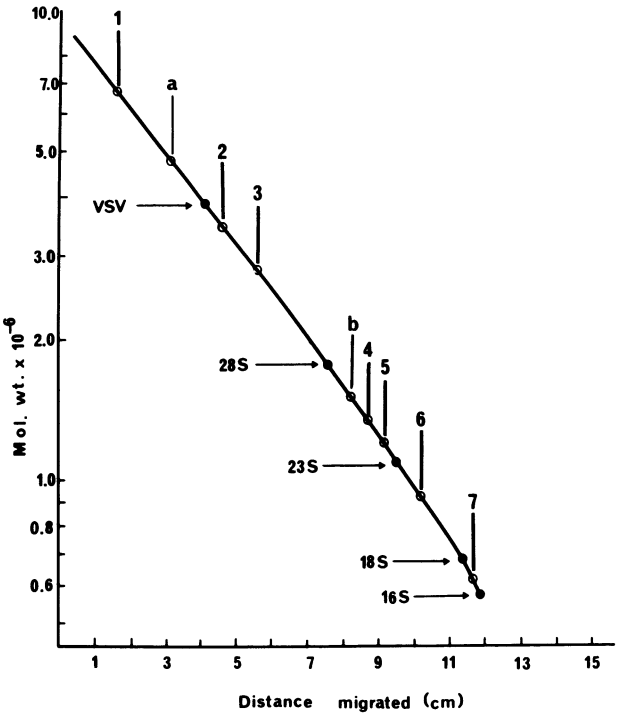
Virion RNA and total viral cytoplasmic RNA

Virion RNA labelled with 3 H uridine was extracted from purified virus and electrophoresed after glyoxal-DMSO treatment. The majority of the virion RNA migrated as a single homogeneous band considerably slower then the genome RNA of vesicular stomatitis virus (Fig. 2 a and b). Virion RNA comigrates with a cytoplasmic RNA species of the same size and the mol. wt. (in millions) of both species was determined to be 6.67. This value is bigger than estimated previously under non denaturing conditions.

For the preparation of cytoplasmic RNA, cells were labelled with 3 H uridine in the presence of actinomycin D for 4 - 6 1/2 h p.i. under single cycle growth conditions. The total cytoplasmic RNA was extracted by phenol SDS. Electrophoresis of total cytoplasmic RNA reproducibly revealed seven major and two minor species (Fig. 2 d). The major RNA species are numbered in order of decreasing size starting with the RNA of genome size as No. 1. The minor species designated with the letter "a" was detected at the same intensity in all preparations of cytoplasmic RNA, whereas the minor species "b" varied to some extend from batch to batch and was not always visible.

An essentially identical RNA pattern was obtained after denaturation of total cytoplasmic RNA with methylmercury hydroxide (Fig. 2 e). This more rigorous chaotropic agent was used for the determination of the mol. wts. of the individual RNA species (Fig. 3 a). The mol. wts. were obtained by coelectrophoresis and mixing of cytoplasmic RNA with radioactively labelled marker RNAs consisting of E.coli 16 S and 23 S RNA, ribosomal 18 S and 28 S L-cell RNA and vesicular stomatitis virion RNA. The standard deviations for each RNA species were obtained by measurements in at least four independent gels and are listed in Fig. 3 b. The values of the mol. wts. determined after glyoxal-DMSO treatment differ only slightly from this figures.

The cytoplasmic RNA pulse labelled early (4 h p.i.) and late(9 h p.i.) during a single growth cycle were analysed. The same number and quantity of RNA species were found. By chromatography on poly U sepharose 50 - 60 % of the cytoplasmic RNA was found to be polyadenylated. Electrophoresis of polyadenylated RNA reveals the same pattern as shown for total cytoplasmic RNA (Fig. 4 b)



RNA No.	MOL. WT. x 10^6
1	6,67 ± 0,46
a	4,70 ± 0,10
2	3,42 ± 0,04
3	2,76 ± 0,05
b	1,50 ± 0,09
4	1,35 ± 0,04
5	1,19 ± 0,04
6	0,93 ± 0,02
7	0,62 ± 0,01

Fig. 3 a) Calibration curve for determination of mol. wts. obtained by electrophoresis of RNAs in 0.9 % agarose-methylmercury hydroxide gels. The following marker RNAs were used: Vesicular stomatitis virus genome (mol. wt. in millions 3.8, Repik and Bishop, 1971), ribosomal 18 S and 28 S RNA from L-cells (mol. wts. in millions 0.68 and 1.74, Loening, 1968) E. coli 16 S and 23 S RNA (mol. wt. in millions 0.55 and 1.07, Stanley & Bock, 1965). The positions of the individual intracellular RNAs relative to the marker RNAs are indicated by vertical bars.
b) Table of mol. wt. of JHM-RNAs.

The RNA species numbered 1 - 7 are single stranded as judged by their sensitivity to digestion with pancreatic RNAse. For this purpose, 32 P labelled cytoplasmic polyadenylated RNA was separated by electrophoresis in polyacrylamide urea gels, cut out after localisation by autoradiography from the gel and reextracted by phenol SDS. The individual RNAs were digested with pancreatic RNAse and ribosomal RNA extracted from the same gels was used as a control. Therefore, these RNA species are most probably polyadenylated single stranded subgenomic mRNAs.

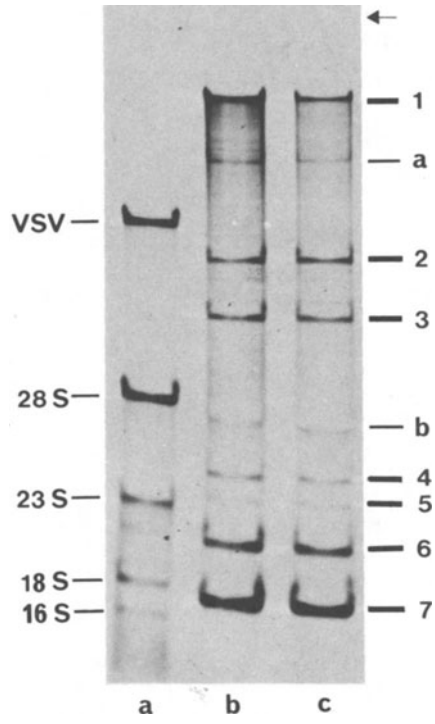


Fig. 4 Fluorogram of JHM-RNA electrophoresed after denaturation with glyoxal-DMSO in 0.9 % agarose
slot a) same marker RNAs as in Fig. 2c,
slot b) polyA selected cytoplasmic JHM RNA
slot c) JHM RNA extracted from polysomes.

Additional support for this conclusion is the association of these RNA species with the polysomes of infected cells. Cytoplasmic extracts obtained by lysis with NP40 were centrifuged into 15 - 40 % sucrose gradients and fractionated. The fractions which sedimented faster than the monosomal 80 S peak were pooled and pelleted through a discontinuous gradient consisting of 2 M and 0.5 M sucrose. Electrophoresis of the RNA extracted from this preparation by phenol SDS revealed no essential difference in comparison to polyadenylated cytoplasmic RNA (Fig. 4 b and c). This polysomal pellet could still contain RNA incorporated into nucleocapsids, which might sediment through 2.0 M sucrose. Therefore, a polysomal pellet was resuspended and analysed on a 15 - 40 % sucrose gradient. As shown in Fig. 5a, the polysomal profile recorded at 260 nm is still conserved. The profile of radioactively labelled RNA showed a typi-

cal bimodal distribution. Analysis of RNA from individual fractions of this polysome gradient by electrophoresis in agarose polyacrylamide urea gels revealed, that most of the genome sized RNA 1 sediments in the region heavier than 200S (Fig. 5b). However, pretreatment of polysomal pellets by EDTA before density gradient centrifugation released all RNA species from the polysomes. Therefore, if nucleocapsids containing RNA 1 are existing in this region of the gradient, these structures might be very fragile and easily falling apart.

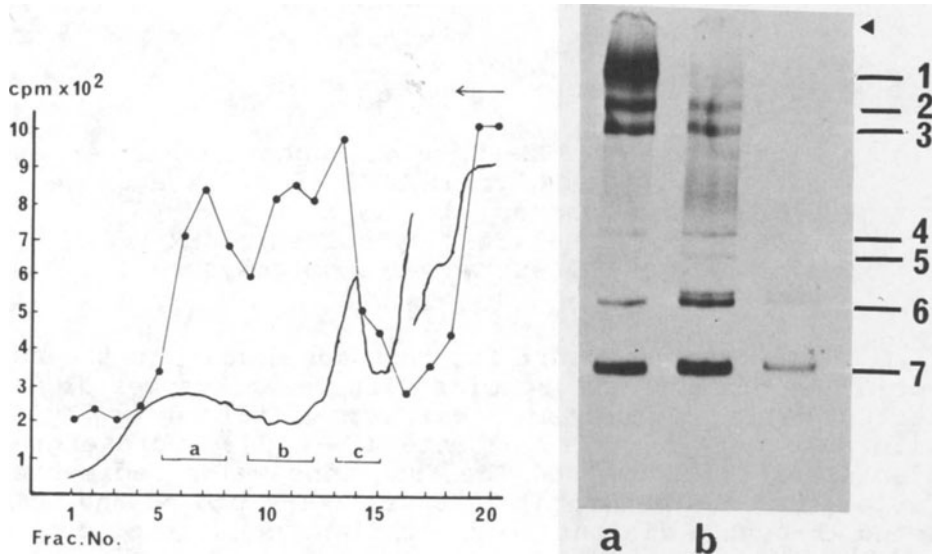


Fig. 5: Sedimentation of polysomes in 15 - 40 % sucrose gradients. a) Distribution of radioactivity (o---o) and optical density at 260 nm (---).b) Electrophoresis in agarose-polyacrylamide urea gels. slot a), b), RNA from the gradient as indicated by bars in Fig. 5a.

CONCLUSION

JHM virus infection of Sac(-) cells leads to the induction of RNA of genome size and six major polyadenylated single stranded subgenomic RNAs. Previous experiments have shown that the smallest RNA codes for a viral protein with a mol. wt. of 60,000 and the next smaller RNA for a viral protein with a mol. wt. of 23,000 (Siddell et al., 1980). Identical results were obtained by translation of RNA 7 (mol. wt. in millions 0.62) and RNA 6 (mol. wt. in millions 0.93) recovered from agarose polyacrylamide urea gels. These results and the translation of other RNA species (Siddell et al., this volume) support the conclusion that coronavirus proteins are synthesized by a set of several independent subgenomic RNAs, which are functionally monocistronic.

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