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## Characterization of Murine Coronavirus RNA by Hybridization with Virus-specific cDNA Probes

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### SUMMARY

Genome RNA of mouse hepatitis virus (MHV) strain A59 has been used as a template to synthesize two virus-specific probes: cDNA<sub>rep</sub>, representing the majority of sequences of the genome RNA and cDNA<sub>3'</sub>, representing the 3' end of the genome RNA. Molecular hybridization with these cDNAs was used to characterize both genome RNA and intracellular virus-specific RNAs. Hybridization of genome RNAs of MHV strains A59, JHM, and MHV-3 with A59 cDNA<sub>rep</sub> showed that, although these three strains exhibit different pathogenicities, they contain closely related nucleotide sequences. Hybridization of intracellular RNA from MHV-infected cells with virus-specific cDNA shows that (i) the majority of virus-specific RNA is polyadenylated, (ii) virus-specific intracellular RNA contains six subgenomic species of the same polarity as genome RNA and (iii) all subgenomic RNAs contain the same 3' sequences as the genome RNA and thus form a nested set of RNAs.

### INTRODUCTION

Coronaviruses cause acute and/or persistent disease in many species of animals. One factor that determines the target organ and lethality of coronavirus infection is the strain of virus (McIntosh, 1974). For example, most strains of mouse hepatitis virus (MHV), such as MHV-3, were isolated from the livers of infected mice and are primarily hepatotropic (McIntosh, 1974). The A59 strain, which is commonly used in the laboratory because it grows to high titres in cell culture, is only weakly pathogenic (Robb & Bond, 1979). The JHM strain is neurotropic and causes persistent demyelinating disease (Bailey *et al.*, 1949). Infection of rodents with this strain has been cited as a model for multiple sclerosis. Most MHV strains are closely related serologically (McIntosh, 1974).

Biochemical studies have been carried out to measure the nucleotide homologies among the genome RNAs of various MHV strains. The A59 and MHV-3 strains, although biologically distinct, possess very similar genome oligonucleotide fingerprints. Fingerprints of other strains, however, show considerable divergence from MHV-3 and A59 (Lai & Stohlman, 1981). The use of oligonucleotide fingerprints to measure homology among genome RNAs has two drawbacks. First, it examines only 10 or 20% of the sequences and second, it is sensitive to small changes, even single base changes, in large oligonucleotides and thus may overemphasize small differences in sequence. Using a cDNA probe representing the nucleocapsid gene of MHV-A59, Cheley *et al.* (1981*b*) found considerable homology between various strains of MHV. This probe, however, represents only 5% of the A59 genome and this 5% (the nucleocapsid gene) is the most likely to be conserved among the strains (Cheley *et al.*, 1981*b*). We have compared the genome RNAs of three MHV strains (A59, JHM and MHV-3) by hybridization with cDNA representing most or all of the A59 genome. Thus, this method presents an advantage over those previously used (oligonucleotide fingerprinting or hybridization with cDNA representing the nucleocapsid gene) in that most or all the sequences of the virus genome may be compared.

The MHV genome is a single-stranded, polyadenylated RNA of  $5.4 \times 10^6$  to  $6.1 \times 10^6$  daltons (Lai & Stohlman, 1978; Leibowitz *et al.*, 1981). During infection of cultured cells, MHV generates six subgenomic cytoplasmic, polyadenylated putative mRNAs that overlap in sequence (Cheley *et al.*, 1981*a*; Jacobs *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1981; Lai *et al.*, 1981; Wege *et al.*, 1981). We have used MHV-specific cDNA probes representing the majority of genome RNA (cDNA<sub>rep</sub>) or specifically the 3' end of genome RNA (cDNA<sub>3'</sub>) in an attempt to locate the site of overlap in the MHV intracellular RNA. Our results are consistent with those of oligonucleotide fingerprinting experiments (Lai *et al.*, 1981), which demonstrate that the MHV subgenomic RNAs contain the 3' sequences of genome RNA and sequences extending various distances toward the 5' end of genome RNA.

## METHODS

*Viruses and cells.* The origin and growth of MHV strains A59 and JHM have been previously described (Robb & Bond, 1979). MHV-3 was obtained from the American Type Culture Collection and grown as described previously (Levy *et al.*, 1981). All viruses were grown in 17CL-1 mouse cells (Sturman & Takemoto, 1972) as previously described (Robb & Bond, 1979). All viruses were plaque-purified twice and stocks grown at a multiplicity of infection (m.o.i.) of approx.  $10^{-4}$ .

### *RNA preparation*

*Preparation of genome RNA.* Cells were infected at m.o.i. of 0.1 to 1 and labelled with inorganic  $^{32}\text{P}$  or [ $^3\text{H}$ ]uridine. MHV was grown at  $37^\circ\text{C}$  and the virus was harvested at 12 to 18 h post-infection. In most experiments, virus was purified from the medium as previously described (Lai & Stohlman, 1978). RNA was extracted from virions by proteinase K treatment in the presence of 1% SDS, followed by phenol extraction (Weiss *et al.*, 1977) and further purified by sedimentation on a sucrose gradient. Genome RNA sedimented as a uniform peak at about 57S. In  $V_{0t}$  experiments, RNA for hybridization was extracted from virus pelleted from the medium, and used without further purification.

*Preparation of intracellular RNA.* Unlabelled RNA was extracted from 17CL-1 cells at various times after infection. Cells were lysed by pipetting in the presence of 1% Nonidet P40 in RSB (0.01 M-Tris-HCl pH 7.4, 0.01 M-NaCl, 0.005 M-MgCl<sub>2</sub>), the nuclei pelleted and RNA extracted from the cytoplasm as described above for virion RNA. In some experiments, poly(A)-containing RNA was selected by chromatography on oligo(dT)-cellulose columns (Aviv & Leder, 1972).

*Agarose gel electrophoresis.* RNA was subjected to electrophoresis in 1% agarose gels containing methylmercuric hydroxide as denaturant (Bailey & Davidson, 1976) and then used in RNA blots as described below.

### *Probe synthesis*

*cDNA<sub>rep</sub>.* Purified A59 genome RNA was used as a template to synthesize high specific activity [ $^{32}\text{P}$ ]dCMP- or [ $^3\text{H}$ ]TMP-labelled cDNA using avian myeloblastosis virus polymerase and oligomers of calf thymus DNA as primers (Taylor *et al.*, 1976; Weiss & Leibowitz, 1981). For liquid hybridization experiments, where a single-stranded cDNA probe is necessary, cDNA<sub>rep</sub> was synthesized in the presence of 100  $\mu\text{g}/\text{ml}$  actinomycin D (Leong *et al.*, 1972) so that the product was 95% single-stranded as assayed by sensitivity to S1 nuclease treatment (data not shown).

*cDNA<sub>3'</sub>.* High sp. act. [ $^{32}\text{P}$ ]dCMP-labelled cDNA was synthesized as above, but using oligo(dT)<sub>12-18</sub> as primer (Tal *et al.*, 1977; Weiss & Leibowitz, 1981). This cDNA, designated cDNA<sub>3'</sub>, was less than 1000 nucleotides in length as assayed by gel electrophoresis (data not shown) and represents not more than 5% of the 3' end of the genome.

### *Molecular hybridization*

*Hybridization in solution.* Liquid hybridization was carried out at  $68^\circ\text{C}$  in 0.6 M-NaCl at the nucleic acid concentrations and for the times indicated in figure legends and assayed by S1 nuclease digestion (Leong *et al.*, 1972). In  $V_{0t}$  curves (Fig. 2), RNA from virus pelleted from increasing amounts of tissue culture medium was hybridized with cDNA to achieve increasing  $V_{0t}$  values where  $V_{0t}$  = volume of medium  $\times$  time of hybridization (Ringold *et al.*, 1975).

*RNA blots.* (i) Dot blots. RNA, at the concentrations shown in figure legends, was spotted onto nitrocellulose filters and hybridized with  $^{32}\text{P}$ -cDNA (Thomas, 1980). (ii) Northern blots. RNA was electrophoresed in agarose gels as above, blotted onto nitrocellulose (Thomas, 1980) and then hybridized with  $^{32}\text{P}$ -labelled cDNA and autoradiographed (Alwine *et al.*, 1977).

## RESULTS

*MHV genome RNA homologies*

The genome RNAs of various strains of MHV were compared by hybridization with a cDNA probe representing the MHV-A59 genome (cDNA<sub>rep</sub>). cDNA<sub>rep</sub> was synthesized and shown to be highly virus-specific as described previously (Weiss & Leibowitz, 1981). Before using this probe as a measure of homology among MHV genome RNAs, the percentage of genome represented in cDNA<sub>rep</sub> was assessed by hybridizing it to <sup>32</sup>P-labelled genome RNA at various ratios of cDNA:RNA and measuring the S1 nuclease-resistant fraction. As shown in Fig. 1, over 65% of genome RNA hybridized to cRNA<sub>rep</sub>. This value is comparable to those obtained with representative probes made with Rous sarcoma virus genome RNA (S. R. Weiss, unpublished data). The fact that saturation of hybridization is obtained at low DNA:RNA ratios illustrates that all sequences are equally represented in the cDNA.

Molecular hybridization with cDNA<sub>rep</sub> was carried out to measure both the amount of virus-specific RNA in virus particles released into the medium above infected cells and the nucleotide homology among genome RNAs of three MHV strains, A59, MHV-3, and JHM. This V<sub>0</sub>t assay (Ringold *et al.*, 1975) is illustrated in Fig. 2. Using the calculations of Ringold *et al.* (1975), a V<sub>0</sub>t<sub>1/2</sub> of 0.1 for A59 RNA indicates the release of approximately  $4 \times 10^9$  physical virus particles (not necessarily infectious) per ml of medium. JHM- and MHV-3-infected cells release 10- to 100-fold fewer particles. This is also true for the amount of infectious virus produced by these three strains of MHV (Robb & Bond, 1979; J. L. Leibowitz, unpublished results). The data illustrated in Fig. 2 also show that these three MHV strains are highly related in nucleotide sequence.

To obtain more quantitative results, various preparations of purified genome and intracellular RNA from the three strains were hybridized with A59 cDNA<sub>rep</sub> under conditions where RNA was in vast excess over DNA (over 1000-fold), hybridization was to very high C<sub>r</sub>t (concentration of RNA  $\times$  time of hybridization) values and plateau levels of hybridization were obtained. These data are summarized in Table 1. All three strains have the majority of sequences in common; the neurotropic JHM strain is the most diverged.

*Intracellular virus-specific RNAs*

Blot hybridization experiments were carried out to measure the accumulation of virus-specific RNA in A59-infected cells as a function of time after infection. In the experiment illustrated in Fig. 3(a), RNA extracted from the cytoplasm of infected cells at various times post-infection was adsorbed to nitrocellulose and hybridized with cDNA<sub>rep</sub> (Thomas, 1980). RNA was detectable at 7 h post-infection and reached a plateau value by 12 h post-infection. By comparison of the dot intensities of intracellular RNA extracted 12 or 24 h post-infection with those of known quantities of purified genome RNA, the percentage of virus-specific RNA in intracellular RNA can be estimated. In Fig. 3(a), hybridization with 5  $\mu$ g of intracellular RNA resulted in a dot of density similar to 0.05  $\mu$ g of purified genome RNA. Thus, viral RNA must be 1% of total intracellular RNA.

RNA extracted at 12 h post-infection was chromatographed on an oligo(dT)-cellulose column to select poly(A)-containing RNA. Both oligo(dT)-binding and non-binding fractions were analysed by hybridization as shown in Fig. 3(b). The poly(A)-containing binding fraction is at least 10-fold enriched for viral sequences. This is in agreement with previous data (Cheley *et al.*, 1981a, b; Leibowitz *et al.*, 1981; Lai *et al.*, 1981; Spaan *et al.*, 1981) that virus-specific intracellular RNA is polyadenylated.

*Hybridization of intracellular RNA with cDNA<sub>3</sub>*

Fig. 4 illustrates agarose gel electrophoresis of RNA extracted from A59-infected cells. After electrophoresis, RNAs were transferred to nitrocellulose and virus-specific RNA was detected by hybridization with virus-specific cDNA<sub>rep</sub> (Fig. 4b). Genome-sized RNA (species 1) and six subgenomic RNAs were detected as described previously (Cheley *et al.*, 1981a; Jacobs *et al.*, 1981; Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1981; Wege *et al.*, 1981). Intracellular

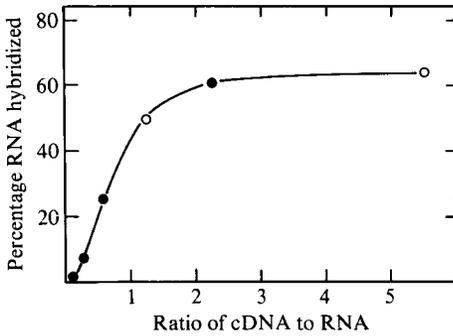


Fig. 1

Fig. 1. The complexity and representativeness of  $cDNA_{rep}$ . Various amounts (0.005 to 0.25 ng) of  $^3H$ - $cDNA_{rep}$  ( $2 \times 10^7$  ct/min/ $\mu$ g) were hybridized to completion with a fixed amount (0.05 ng) of  $^{32}P$ -labelled genome RNA (500 ct/min,  $10^7$  ct/min/ $\mu$ g). Hybridization was assayed by digestion with S1 nuclease. The symbols represent two different experiments.

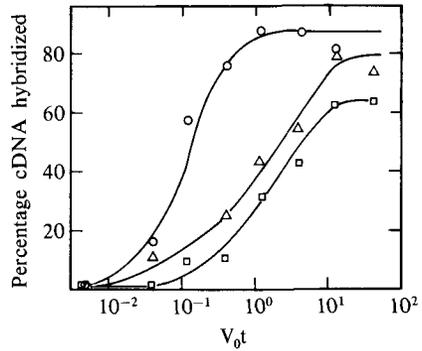


Fig. 2

Fig. 2.  $V_0t$  analysis of MHV-infected cells. RNA was extracted from virions pelleted from the medium above cells infected with A59 (O), MHV-3 ( $\Delta$ ) and JHM ( $\square$ ). RNA from increasing amounts of medium was hybridized to  $^3H$ - $cDNA_{rep}$  (2000 ct/min,  $2 \times 10^7$  ct/min/ $\mu$ g). Hybridization was for 16 h and was assayed by S1 nuclease digestion.

Table 1. Homology among the A59, MHV-3 and JHM coronavirus genomes

Source of RNA	Percent hybridization of A59 $cDNA_{rep}$ *
A59	100
MHV-3	90
JHM	74

\* A59  $cDNA_{rep}$  was hybridized to completion with a vast excess of RNA purified from virions or infected cells. These values are averages of three experiments and have been normalized to 100% hybridization of A59  $cDNA_{rep}$  with its homologous A59 RNA. The actual values for hybridization of A59  $cDNA_{rep}$  with A59 RNA ranged from 85 to 100%.

RNA was also hybridized with a cDNA which represents the 3' end of the genome,  $cDNA_3'$  (Weiss & Leibowitz, 1981). All seven RNAs were detected by hybridization with  $cDNA_3'$  (Fig. 4a) as well as with  $cDNA_{rep}$  (Fig. 4b). This is consistent with the oligonucleotide fingerprint results of Lai *et al.* (1981), that show that all these intracellular RNAs overlap at their 3' ends. The larger RNAs (1, 2 and 3) hybridize less well with  $cDNA_3'$ , than with  $cDNA_{rep}$  (compare a and b), as might be expected for a probe that measures molar amounts of RNA rather than total mass.

#### DISCUSSION

The A59, JHM, and MHV3 strains of MHV have different biological properties. MHV3 is primarily hepatotropic (McIntosh, 1974), JHM is neurotropic (Bailey *et al.*, 1949) and A59 is weakly pathogenic (Robb & Bond, 1979). We have used  $cDNA_{rep}$ , synthesized from A59 genome RNA, to compare the genomes of these strains. This cDNA is highly virus-specific (Weiss & Leibowitz, 1981) and represents the majority of the genome (Fig. 1). Thus, hybridization with  $cDNA_{rep}$  is a better measure of genome homology than oligonucleotide fingerprinting (Lai & Stohlman, 1981), or hybridization with a cDNA representative only of the nucleocapsid gene (Cheley *et al.*, 1981b), both of which measure only a small percentage of genome RNA sequences. Molecular hybridization studies with  $cDNA_{rep}$  suggest that these strains are highly related in sequence, with JHM being the most diverged. Our results are in basic agreement with results of oligonucleotide fingerprint maps of these genome RNAs (Lai & Stohlman, 1981; Leibowitz *et al.*, 1981; Weiss & Leibowitz, 1981). They also show a greater

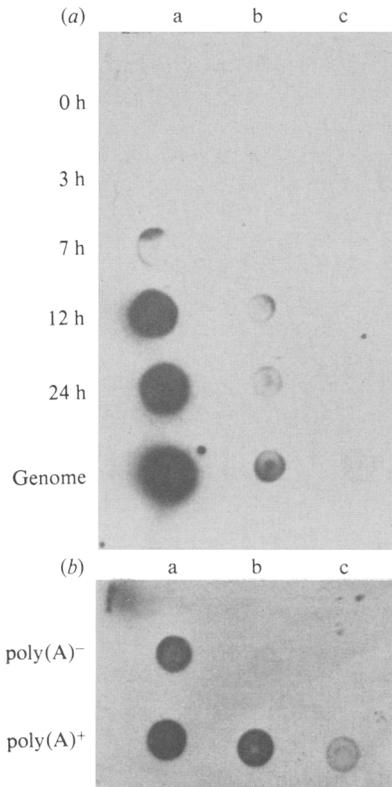


Fig. 3

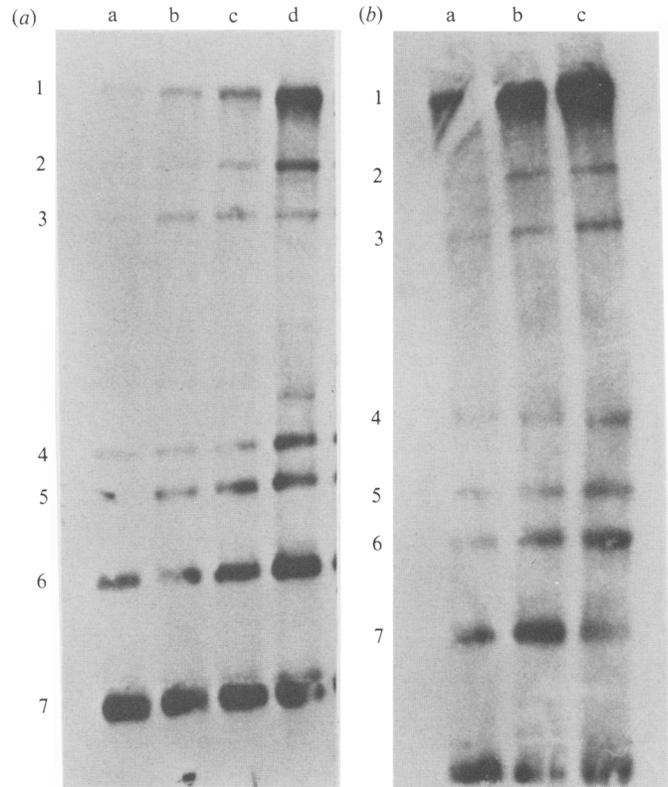


Fig. 4

Fig. 3. Kinetics of accumulation and polyadenylation of A59 intracellular RNA. (a) RNA was extracted from 17CL-1 cells at various times after infection by A59 RNA, at three dilutions [(a) 5 µg/3 µl, (b) 0.5 µg/3 µl, (c) 0.05 µg/3 µl] was spotted onto nitrocellulose, and hybridized for 24 h with  $^{32}\text{P}$ -cDNA<sub>rep</sub> ( $3 \times 10^5$  ct/min,  $10^8$  ct/min/µg). Genome RNA [(a) 50 ng/3 µl; (b) 5 ng/3 µl; (c) 0.5 ng/3 µl] was used as a control. (b) RNA extracted 12 h post-infection was chromatographed on an oligo(dT)-cellulose column. Equal fractions of the binding and non-binding RNAs were analysed as in (a). Hybridization was for 24 h with  $^{32}\text{P}$ -cDNA<sub>rep</sub> ( $3 \times 10^5$  ct/min,  $10^8$  ct/min/µg).

Fig. 4. Hybridization of cDNA<sub>3</sub> with intracellular A59 RNAs. 17CL-1 intracellular RNA (extracted 12 h post-infection) was electrophoresed in a denaturing agarose gel, transferred to nitrocellulose paper and hybridized with  $^{32}\text{P}$ -cDNA. (a) Hybridization with cDNA<sub>3</sub> ( $3 \times 10^5$  ct/min,  $10^8$  ct/min/µg) was for 24 h: (a) 2 µg, (b) 4 µg, (c) 5 µg and (d) 10 µg RNA. (b) Hybridization with cDNA<sub>rep</sub> ( $7 \times 10^5$  ct/min,  $10^8$  ct/min/µg) was for 24 h: (a) 2 µg, (b) 4 µg and (c) 5 µg RNA. The gels shown in (a) and (b) contained RNA samples from the same preparation and were run in parallel.

degree of homology between MHV-3 and A59 than shown by hybridization with cDNA representing the nucleocapsid gene (Cheley *et al.*, 1981*b*).

Molecular hybridization may be used to detect and quantify virus-specific RNA in MHV-infected cells (Fig. 3) or in physical virus particles released from cells (Fig. 2). This technique has an advantage over measuring virus-specific RNA by monitoring incorporation of [ $^3\text{H}$ ]uridine into trichloroacetic acid-precipitable counts in the presence of actinomycin D, because hybridization is highly virus-specific and much more sensitive. Hybridization can detect less than one copy of virus-specific genome RNA per cell (Parker & Stark, 1979; Brahic & Haase, 1978) and low levels of physical virus particles released into the medium (Ringold *et al.*, 1975). This will be especially useful in the analysis of MHV persistence in cells or in animals, where only small amounts of viral nucleic acids may be present.

From the dot blot experiment (Fig. 3) it was estimated that virus-specific RNA represents approximately 1% of total intracellular RNA during lytic infection in cultured cells. This agrees with our previous estimate from solution hybridization. Intracellular RNA hybridized to

cDNA<sub>rep</sub> with a  $C_{r1/2}$  of between 1 and 10, indicating the presence of approximately 1000 MHV genome-equivalents per cell (Weiss & Leibowitz, 1981). This is similar to the number estimated by Cheley *et al.* (1981*a, b*) to be present in L-cells infected by MHV. However, the  $C_{r1/2}$  values of 0.05 to 0.16 obtained by these authors differ from ours. We do not understand the discrepancy.

MHV generates multiple, subgenomic intracellular polyadenylated putative mRNAs (Cheley *et al.*, 1981*a*; Jacobs *et al.*, 1981; Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1981; Wege *et al.*, 1981). These RNAs were shown to have sequences in common with genome RNA as evidenced by overlap in their oligonucleotide fingerprint maps (Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1981) and by their hybridization to cDNA representing the nucleocapsid gene (Cheley *et al.*, 1981*a*). These experiments, however, did not locate the site of the overlapping sequences. Our hybridization experiment with cDNA<sub>γ</sub> (Fig. 4), in addition to the oligonucleotide fingerprinting experiments of Lai *et al.* (1981), show the overlapping sequences present in all the subgenomic mRNAs are present at the 3' end of genome RNA.

The synthesis of a nested set of mRNAs is common to several classes of RNA viruses (Cancedda *et al.*, 1974; Weiss *et al.*, 1977). In most of these cases, each subgenomic RNA serves as mRNA for its 5' gene. We (Leibowitz *et al.*, 1982) and others (Cheley *et al.*, 1981; Rottier *et al.*, 1981; Siddell *et al.*, 1980) have started to analyse the mRNA capacities of the MHV intracellular RNAs. In cell-free translation experiments, RNAs 3 and 6 have been shown respectively to code for E2 and E1 (both structural glycoproteins), and RNA 7 for N (nucleocapsid structural protein). The sizes of the proteins translated from these mRNAs are, in each case, consistent with translation starting in the 5' non-overlapping part of the RNA. However, this has yet to be proved.

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