JOURNAL OF VIROLOGY, May 1978, p. 236-242
0022-538X/78/0026-0236$02.00/0

Vol. 26, No. 2
Printed in U.S.A.

RNA of Mouse Hepatitis Virus

MICHAEL M. C. LAI AND STEPHEN A. STOHLMAN†*

Departments of Microbiology and Neurology, University of Southern California School of Medicine, Los Angeles, California 90033

Received for publication 28 December 1977

The RNA of mouse hepatitis virus, a coronavirus, was isolated from the virus released early in the infection and analyzed by sucrose gradient sedimentation and electrophoresis. It was found to consist of a piece of single-stranded RNA of about 60S. Its molecular weight was estimated to be 5.4×10^6 by electrophoresis in methylmercury-agarose gels. At least one third of the RNA contained polyadenylated sequences. It is, therefore, probably positive stranded. The virus harvested late in the infection contained, in addition to 60S, some 30 to 50S RNA that are possibly degradation products of the 60S RNA. No difference in the electrophoretic behavior could be detected between the RNA isolated from a pathogenic (JHM) and a nonpathogenic (A_{59}) strain.

Coronaviruses are ubiquitous agents associated with disease states in a variety of mammalian and avian hosts (18) and are classified as a separate taxonomic group (29). The classification is based mainly on morphological evidence of club-shaped surface projections and maturation by budding from internal cellular membranes but not from plasma membranes. Although a number of diverse agents have been included in this taxonomic group, only recently have attempts been made to study their molecular and biophysical properies (2, 7, 8, 10, 11, 16, 17, 22, 23, 26, 30).

Mouse hepatitis virus (MHV) has been classified as a member of this group (28). It occurs primarily as a natural, latent infection of mice (6) and has been isolated from a variety of laboratory strains of mice. Although antigenically similar (3, 28), different MHV strains differ widely in biological properties in vivo. Most cause a fatal hepatitis in mice; however, one is pathogenic only for nude mice (12), and two are able to establish chronic neurological disease (9, 29).

In this paper we describe studies of the genome RNA of two types of MHV, one a nonpathogenic strain (A_{59}), and the other a pathogenic strain (JHM). JHM causes acute encephalomyelitis with both acute and chronic demyelination in mice following intracranial inoculation (9, 31).

MATERIALS AND METHODS

Viruses and cells. The A_{59} strain of MHV, obtained from C. Bond (University of California, San Diego), was used in this study following four passages in DBT cells, a continuous mouse cell line derived from a brain tumor (S. A. Stohlman and L. P. Weiner, Arch. Virol., in press). A large-plaque variant of the neurotropic JHM strain of MHV (MHV-4) (31) was purified from the eighth passage in suckling mouse brain (31; Stohlman and Weiner, Arch. Virol., in press) by five consecutive plaque isolations in DBT cells. This mutant, designated DL, was further passaged three times in DBT cells and checked for uniformity of plaque morphology on both DBT and 17 clone 1 (17CL1) cells (24) before being used in this study.

Virus growth and radiolabeling. A_{59} was adsorbed to 17CL1 monolayers in 1,300-cm^2 roller bottles for 60 min at 37°C at a multiplicity of infection of 10.0. The monolayers were subsequently washed three times with Eagle minimum essential medium, and 30 ml of minimum essential medium containing 5% dialyzed fetal calf serum and 30 μCi of [^{3}H]uridine or [^{3}H]adenosine (Amersham/Searle, specific activity 47 Ci/mmol) per ml was added immediately after adsorption. Radiolabeling with [^{32}P]orthophosphate (Amersham/Searle) was done in phosphate-free Ham F-10 medium at 300 μCi/ml. Radiolabeled JHM virus was prepared by infecting monolayers of DBT cells in roller bottles with a multiplicity of infection of 0.5. [^{3}H]uridine or [^{32}P]orthophosphate was added immediately following adsorption as described above for A_{59}.

Fluids from all infected cultures were harvested before significant cellular destruction occurred. 17CL1 cultures infected with A_{59} or, in some experiments, JHM, were harvested at 16 to 18 h postinfection. DBT cultures were harvested at 14.5 h postinfection.

Purification of viruses. Supernatant fluids were first clarified by centrifugation at $1,200 \times g$ for 5 min and $15,000 \times g$ for 30 min at 4°C. Virus was concentrated by pelleting at 25,000 rpm in an SW27 rotor for 2 h at 4°C. The pellets were resuspended in TMEN buffer (containing 50 mM Tris-malate, pH 6.0, 1 mM EDTA, and 100 mM NaCl) and gently homogenized with a Dounce homogenizer. The virus suspension was then layered onto a discontinuous gradient contraining 5 ml of 30% sucrose and 5 ml of 50% sucrose in TMEN

† Reprint requests should be sent to: 142 McKibben, 2025 Zonal Avenue, Los Angeles, CA 90033.

and centrifuged at 27,000 rpm in an SW27 rotor for 3 h at 4°C. The visible band at the 30–50% sucrose interface was removed by side puncture of the tube and either used immediately (see Results) or diluted with TMEN and recentrifuged on either a linear 15 to 50% sucrose or a 15 to 40% potassium tartrate gradient at 27,000 rpm in an SW27.1 rotor for 18 h at 4°C. Fractions were collected from the bottom of the centrifuge tube.

Extraction and sedimentation analysis of viral RNA. Purified virus in sucrose was diluted with 3 volumes of NTE buffer (10 mM Tris-hydrochloride, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and then disrupted with 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol. The RNA was extracted twice with NTE buffer-saturated phenol and then precipitated with 2 volumes of ethanol at −20°C overnight. The RNA was pelleted by sedimentation at 20,000 × *g* for 15 min and then used for sucrose gradient sedimentation or gel electrophoresis.

Sucrose gradient sedimentation was performed in linear sucrose gradients made up of 10 to 25% sucrose containing NTE buffer and 0.1% SDS. The RNA samples were dissolved in 0.3 ml of NTE buffer containing 0.2% SDS and centrifuged in an SW41 rotor at 40,000 rpm for 3.5 h at 20°C. The 70S and 35S RNA of Rous sarcoma virus and 28S rRNA from chicken fibroblasts were included as markers.

Electrophoresis. Polyacrylamide gel electrophoresis of the RNA was done by a modification of the method of Duesberg (5). The RNA was dissolved in 40 μl of sample buffer containing 4 mM Tris-acetate (pH 7.0), 2 mM sodium acetate, 1 mM EDTA, 0.1% SDS, and 10% glycerol and subjected to electrophoresis in 2% polyacrylamide gels cross-linked with bisacrylamide (7 by 0.6 cm). Electrophoresis was performed at 50 V for 6 h. After electrophoresis, gels were frozen and sliced into 1-mm fractions. Fractions were incubated in toluene-based scintillation fluid containing 10% NCS (Amersham/Searle) and 1% water at 50°C for 5 h prior to counting.

Methylmercury hydroxide-agarose gel electrophoresis was adapted with minor modifications from the method of Bailey and Davidson (1). Briefly, the RNA samples were dissolved in 40 μl of 0.5 × ER buffer (ER buffer: 50 mM boric acid, 5 mM sodium borate, 1 mM EDTA, and 10 mM sodium sulfate, pH 8.19) containing 5 mM methylmercury hydroxide and 5% sucrose. Electrophoresis was carried out in 1% agarose gels containing 5 mM methylmercury hydroxide at 5 mA per gel for 5 h. After electrophoresis, the gels were sliced into 1-mm fractions and counted in Brays scintillation fluid.

RNA binding to nitrocellulose filters. The binding of polyadenylate [poly(A)]-containing RNA to nitrocellulose (Millipore) filters was performed by a modification of the method described by Lee et al. (15). The [^{3}H]uridine- or ^{32}P-labeled RNA samples were dissolved in 50 μl of buffer containing 10 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA. Ten volumes of ice-cold binding buffer containing 10 mM Tris-hydrochloride (pH 7.4), 500 mM KCl, and 1 mM $MgCl_2$ were added to each sample. After 10 min at 0°C, the poly(A)-containing RNA was collected on filters prewashed with binding buffer. Filters were dried before counting.

RESULTS

Virus purification. [^{3}H]uridine-labeled A_{59} virus was harvested from 17CL1 cultures at 16 to 18 h postinfection, well before the time of maximum virus titer and maximum cytopathology (24 to 30 h postinfection). The virus isolated from the 30–50% sucrose interface was further centrifuged on a 15 to 50% sucrose gradient. A single band of radioactivity and infectivity was found at the density of 1.18 gm/cm^3 (Fig. 1a). In contrast, virus harvested at the time of maximum yield (24 to 30 h) contained two bands in sucrose gradients at densities of 1.18 and 1.23 gm/cm^3 (Fig. 1b). The infectivity of the virus at 1.23 gm/cm^3 was at least 100-fold lower than that of the virus at 1.18 gm/cm^3 (Fig. 1b). Furthermore, the virus purified from the culture early in the infection could be converted by incubation with 2% Nonidet P-40 at room temperature for 5 min into a heterogeneous band with an average density of 1.23 gm/cm^3. This treatment also reduced the virus titer by over 99%. Therefore, we consider the band at 1.23 gm/cm^3 to represent a degradation product of the virion. Essentially similar results were obtained with JHM virus harvested early (14.5 h) or late (18 h) postinfection from DBT cultures.

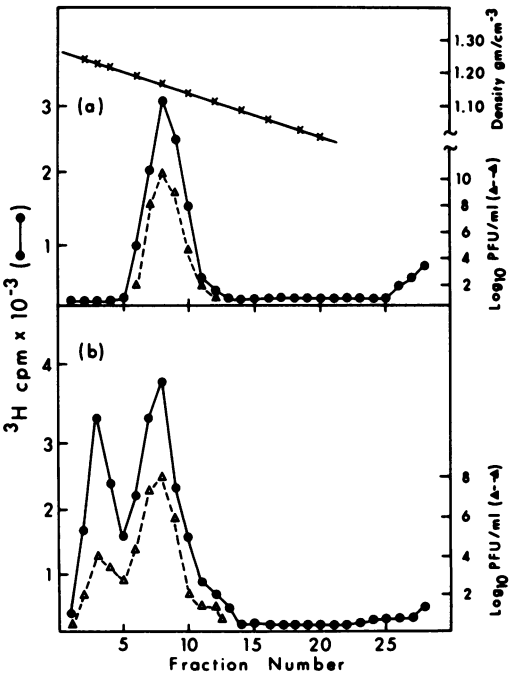

FIG. 1. *Sucrose density gradient purification of A_{59} virus. (a) Purification of virus from 17CL1 cells 14 h postinfection. (b) Purification of virus from 17CL1 cells 24 h postinfection.*

Sedimentation analysis of viral RNA. The [^{3}H]uridine-labeled nucleic acids of the virus were extracted twice with SDS-phenol and then precipitated with 2 volumes of ethanol. The nucleic acids were dissolved in NTE buffer and analyzed by sedimentation through 10 to 25% sucrose. The majority of [^{3}H]uridine-labeled MHV RNA sedimented as a single peak (Fig. 2a). This result was found with virus purified either early or late in the infection. The ^{32}P-labeled 70S RNA of Rous sarcoma virus (RSV; Prague strain of subgroup C) and its heat-dissociated 35S subunit were included as sedimentation velocity markers. The A_{59} RNA sedimented between these two markers with a sedimentation rate estimated to be about 60S.

To determine whether the secondary structure affected its sedimentation behavior, A_{59} RNA was heated at 100°C for 1 min and then again analyzed by sucrose gradient sedimentation. No change in sedimentation rate was noted when the RNA from virus purified early in the infection was analyzed; there was no evidence of subunit structure (Fig. 2b). In contrast, the RNA extracted from virus harvested later in the infection showed heterogeneity after heat denaturation (Fig. 2c). In addition to the main 60S RNA, there were variable amounts of smaller RNA species with sedimentation rates between 30 and 50S. The amount of these RNA species varied from preparation to preparation and also varied with the method of virus purification. They were particularly prominent when the virus was purified in potassium tartrate equilibrium gradients. Since the virus harvested late in the infection contained degraded virus particles and was less infectious (Fig. 1b), these smaller RNA species (30 to 50S) are probably degradation products of the 60S viral RNA. To minimize possible degradation artifacts, the virus was only purified to the 30–50% sucrose cushion step for most of the studies reported here.

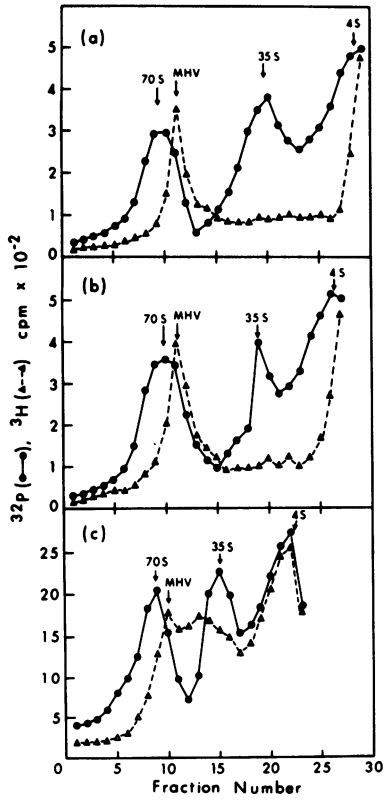

FIG. 2. *Sucrose gradient sedimentation of MHV (A_{59}) RNA. (a) Native RNA of MHV harvested at 16 h postinfection. (b) MHV RNA heated at 100°C for 1 min (early harvest). (c) Heat-denatured RNA of MHV harvested at 24 h postinfection. Sedimentation was carried out in 10 to 25% sucrose gradients at 40,000 rpm for 3.5 h at 20°C in an SW41 rotor. ^{32}P-labeled 70S and 35S RNAs RSV (Prague strain of subgroup C) were included as markers.*

In addition to the 60S RNA, a variable amount of 4S RNA was also found in MHV preparations irrespective of harvest time. This RNA migrated as a homogeneous 4S RNA species in 10% polyacrylamide gels (data not shown). It is not clear, however, whether it represents contaminated cellular tRNA or is a specific component of the virus.

Electrophoretic studies. Polyacrylamide gel electrophoresis was carried out to provide further insight into the size and structure of MHV RNA. The MHV RNA migrated slightly faster than 70S, but much slower than 35S RNA of RSV (Fig. 3a). No smaller RNA species were visible, and the electrophoretic mobility of the major MHV RNA did not change even when the RNA was heat denatured at 100°C for 1 min (data not shown). However, variable amounts of the RNA with higher electrophoretic mobility than the major MHV RNA was present in preparations from late-harvested virus. These smaller RNA species were particularly prominent when the RNA was heat denatured, although the electrophoretic mobility of the major RNA species remained the same (Fig. 3b). These smaller RNAs probably represent the degradation products of the genome RNA, as discussed in the previous section.

Since the MHV RNA has a sedimentation value and electrophoretic mobility very close to those of the 70S RNA of RSV (Fig. 1 and 2), its molecular weight could be estimated to be about 5×10^6 to 6×10^6 if the 70S RNA is assumed to be 6×10^6 to 7×10^6 (4, 13, 19). This value was further tested by electrophoresis in agarose gels containing methylmercuric hydroxide (1). RNA

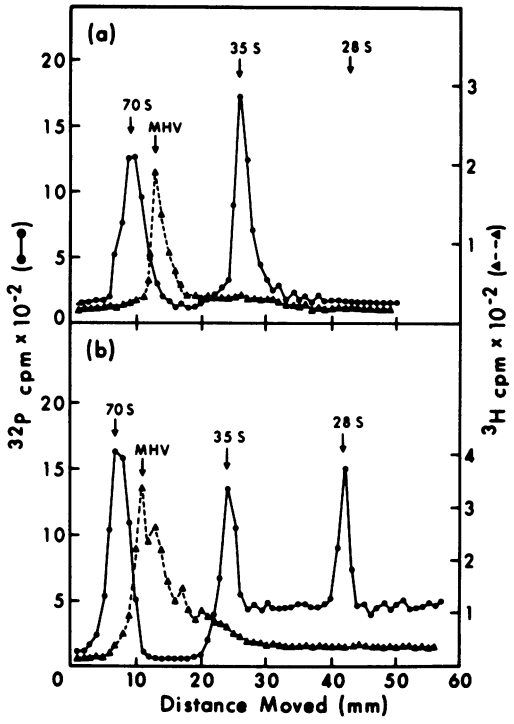

FIG. 3. *Polyacrylamide gel electrophoresis of MHV RNA. (a) Native RNA of MHV harvested 16 h postinfection. (b) Heat-denatured RNA of MHV harvested at 24 h postinfection. Electrophoresis was performed in 2% polyacrylamide gels cross-linked with bisacrylamide at 5 mA per gel for 6 h. 70S and 35S RSV RNA and 28S rRNA were included as markers.*

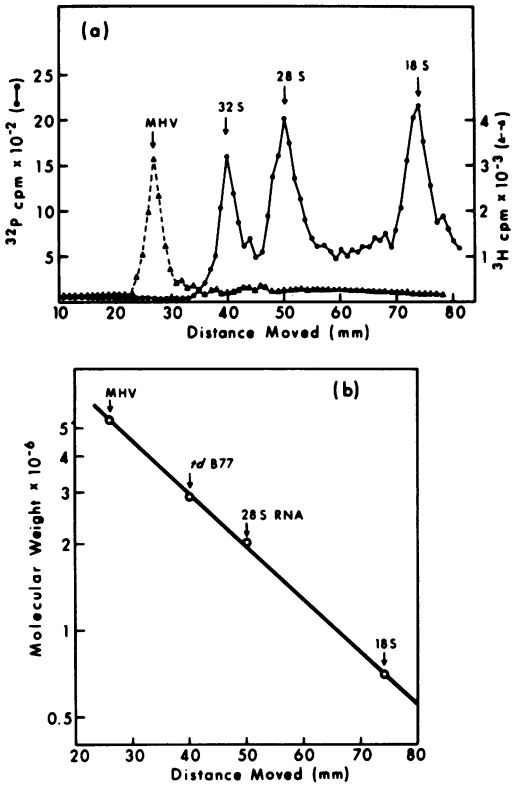

FIG. 4. *Methylmercury-agarose gel electrophoresis of MHV RNA. (a) MHV RNA (early harvest), RNA of the transformation-defective B77 strain in RSV (molecular weight 3 × 10^6), 28S rRNA (molecular weight 2 × 10^6) and 18S rRNA (molecular weight 0.7 × 10^6) of chicken fibroblasts were co-electrophoresed in a 1% agarose gel containing 5 mM methylmercury hydroxide at 5 mA per gel for 5 h. (b) Plot of logarithms of molecular weight versus electrophoretic mobilities of the RNAs.*

is completely denatured under this condition, and logarithms of the molecular weights are linear with respect to electrophoretic mobility (1). Again, the MHV RNA migrated as a single peak (Fig. 4a), and its molecular weight was estimated to be 5.4 × 10^6 (Fig. 4b).

Single-strandedness and presence of poly(A) in MHV RNA. The finding that denaturation by either heat or methylmercury did not alter the sedimentation rate and electrophoretic mobility of MHV RNA suggests that it is single stranded. To provide further proof, we also subjected MHV RNA to digestion with RNase. [^{3}H]uridine-labeled MHV RNA was completely sensitive to RNase even in the presence of high salt (0.3 M NaCl) (Table 1). Under the same conditions, the single-stranded RNA genome of RSV and 28S rRNA were digested to the same extent. This is further suggestive evidence that MHV RNA is single stranded. However, when [^{3}H]adenosine-labeled MHV RNA was treated under the same conditions, 12% of the counts were resistant. This property is similar to RSV RNA, which contains adenylic acid-rich sequences (14), and suggested the possibility

TABLE 1. *RNase treatment of MHV RNA*[a]

RNA (label)	Total cpm	Trichloroacetic acid-insoluble cpm after RNase	Percent RNase resistance
A_{59} ([^{3}H]uridine)	12,469	179	1.4
A_{59} ([^{3}H]adenosine)	13,515	1,654	12.2
RSV ([^{3}H]uridine)	8,976	101	1.1
28S ([^{3}H]uridine)	43,010	269	0.7

[a] RNA samples were dissolved in 200 μl of a solution containing 0.3 *M* NaCl and 0.03 *M* sodium citrate (pH 7.0). They were heated at 100°C for 1 min, quenched at 0°C, and then incubated with RNase A (20 μg/ml) and RNase T1 (50 U/ml) at 37°C for 30 min. Trichloroacetic acid-insoluble counts per minute (cpm) were collected on nitrocellulose filters and counted in toluene-based liquid scintillation fluid.

that MHV RNA might also contain polyadenylated sequences.

Since poly(A)-containing RNA binds to nitrocellulose filters in the presence of high salt concentrations (14, 15), the binding of MHV RNA was examined. Only about 33% of the native RNA was bound (Table 2). By contrast, 100% of the 70S RNA of RSV was bound. We concluded that only one third of the MHV RNA contained poly(A). To rule out the possibility that the partial binding was due to nonspecific degradation of the RNA, we separated MHV RNA by neutral sucrose gradient as shown in Fig. 1. The 60S RNA was isolated and tested for its ability to bind to nitrocellulose filters. Again, only about 33% of the RNA was bound. The same finding was obtained when oligodeoxythymidylic acid-cellulose columns were used for detection of poly(A) (data not shown). Thus, only about one third of MHV RNA contained polyadenylated sequences long enough to bind to nitrocellulose filters. Whether the rest of the RNA contains any poly(A) sequences or not remains to be investigated.

Comparison of the RNAs of pathogenic and nonpathogenic MHV. The data presented above were obtained from studies of the nonpathogenic A_{59} strain of MHV. Preliminary evidence indicates that A_{59} and the neurotrophic JHM strain differ in their antigenicity and protein compositions (Stohlman, unpublished data). To see whether these two strains of MHV differ in their RNA structure, we compared their electrophoretic mobility in polyacrylamide gels. No detectable difference was found by co-electrophoresis of ^{32}P-labeled JHM RNA and [^3H]-uridine-labeled A_{59} RNA (Fig. 5). This was true for both native and denatured RNAs of these two strains.

DISCUSSION

The nucleic acid extracted from purified MHV was found to be a single-stranded RNA of approximately 60S by sucrose gradient centrifugation. The molecular weight of this RNA was estimated to be about 5.4×10^6 by electrophoresis in methylmercury-agarose gels. This finding is similar to recent reports on the RNA purified from infectious bronchitis virus (IBV) (17, 21, 30), the prototype coronavirus (28). MHV RNA contained poly(A) sequences and is, therefore, probably a positive-stranded RNA. This conclusion is consistent with the report that MHV RNA is infectious (L. S. Sturman, personal communication). IBV RNA has also recently been found to be infectious (16, 21). However, only about one third of the RNA bound to nitrocellulose filters, which suggests that not all of the MHV RNA contains poly(A) tracts. Alternatively, all of the MHV RNA might contain poly(A) tracts, some of which are, however, too short to bind to nitrocellulose filters. We are unable to distinguish between these two possibilities at the present time. An analysis of the size of the poly(A) tract is inconclusive (unpublished data). Poly(A) tracts have also been detected on only 30 to 83% of the RNA isolated from IBV, and the IBV RNA is infectious (16, 21).

The homogeneity of MHV RNA was found to be dependent upon the time of virus harvest. Heat denaturation of RNA from virus harvested early (16 to 20 h postinfection) did not affect the sedimentation pattern, whereas heat denaturation of RNA from virus harvested at the time of maximum virus yield resulted in heterogeneous RNA of 30 to 50S. Our data showed that the late-harvested virus was heterogeneous and possibly contained degraded virus particles that had reduced specific infectivity (Fig. 2). Therefore, the heterogeneity of the RNA in the late-harvested virus detected after heat denaturation was probably due to degradation of the 60S RNA after virus maturation. This may help

TABLE 2. *Binding of RNAs to nitrocellulose filters*

RNA (label)	Total cpm[a]	cpm Bound	Percent binding
MHV ([^3H]uridine)	7,507	2,511	33
MHV ([^3H]adenosine)	4,150	1,581	38
60S MHV ([^3H]uridine)[b]	2,800	951	34
70S RSV ([^3H]uridine)	9,391	9,510	100
28S rRNA ([^3H]uridine)	36,415	1,054	2.9

[a] cpm, Counts per minute.

[b] 60S RNA of A_{59} was isolated from the sucrose gradient sedimentation of the total A_{59} RNA as described in Fig. 2a.

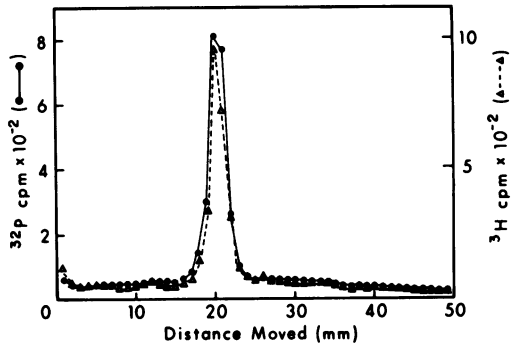

FIG. 5. *Polyacrylamide gel electrophoresis of A_{59} and JHM RNA. ^{32}P-labeled A_{59} RNA and [^3H]uridine-labeled JHM RNA were heat denatured at 100°C for 1 min and then co-electrophoresed in 2% polyacrylamide gel at 5 mA per gel for 6 h.*

explain the finding that the RNA from human coronavirus OC-43 was dissociated into heterogeneous RNAs after heat denaturation (26). An earlier report also suggested that IBV RNA was heterogeneous (25), although it has recently been shown to be a heat-stable homogeneous RNA (17).

Two reports suggested that coronavirus RNA cosedimented with the RNA of retroviruses and dissociated into 35S RNA subunits with heat denaturation (2, 26). Activation of a retrovirus in porcine cells inoculated with porcine coronaviruses (8) has been suggested to account for these findings (21) and may also explain the results obtained with OC-43 purified from mouse brain (26). Indeed, we have found that infection of 17CL1 cells with JHM, but not with A_{59}, resulted in the isolation of virions with RNA of approximately 70S, which was denatured by heat into subunits of approximately 35S (Lai and Stohlman, unpublished data). The similarity in size and density of retrovirus and coronavirus particles and the possible activation of retroviruses by coronaviruses may lead to confusion in understanding the molecular biology of coronaviruses.

Purified MHV has only four structural proteins (23), two of which appear to be derived from the same gene (22). Therefore, the virus particles contain a total of approximately 260,000 daltons of structural proteins, which can account for only about 40% of the coding capacity of the viral genome. This suggests either that a large number of nonstructural proteins are synthesized during infection or that the expression of the entire genome is not required for production of infectious virus. Expression of normally repressed gene functions may take place during latent infections in vitro (20; K. V. Holmes and R. Allen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S396, p. 345; Stohlman and Weiner, Arch. Virol., in press) or in vivo (8, 9, 29).

ACKNOWLEDGMENTS

This investigation received financial support from Public Health Service grants 1F32-NS-05410 and RO1-NS-12967 from the National Institute of Neurological and Communicable Disorders and Stroke.

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