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## Defective Interfering Particles of Mouse Hepatitis Virus

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*Received June 6, 1983; accepted October 27, 1983*

After six to eight serial undiluted passages of mouse hepatitis virus (JHM strain) in DBT cell culture, a decrease in the yield of infectious virus occurred, and with further passages fluctuating yields of infectious virus were observed. The serially passaged virus interfered with the multiplication of the standard JHM virus, but not with vesicular stomatitis virus. After sucrose equilibrium centrifugation of high passage virus, a single peak contained both infectious virus and interfering activity. This virus population resembled the original JHM virus in its structural proteins, but it contained an increased proportion of a protein with a molecular weight of  $65 \times 10^6$ . Genomic RNA from standard JHM virus contained a single species of RNA with a molecular weight of  $5.4 \times 10^6$ . After five undiluted passages, however, the virion population contained two RNA species with molecular weights of  $5.4 \times 10^6$  and  $5.2 \times 10^6$ . RNase T<sub>1</sub> resistant oligonucleotide fingerprinting of these RNAs showed that the lower molecular weight RNA had lost several oligonucleotide spots that were present in the genomic RNA of the standard JHM virus. After several serial diluted passages of passage 10 virus, a single virus population was obtained which again had only standard virus RNA with a molecular weight of  $5.4 \times 10^6$  and lacked interfering activity. These results indicated that defective interfering particles were generated by serial undiluted passages of JHM virus.

### INTRODUCTION

Defective interfering (DI) particles have been described in most virus groups (Holland *et al.*, 1980; Huang and Baltimore, 1977). Characteristically DI particles (a) lack part of the viral genome, (b) possess a normal complement of structural proteins, (c) replicate only with the help of standard virus, (d) interfere with replication of the homologous virus. Many studies have focused on the role of DI virus genomes in virus evolution and their involvement in persistent infection (Friedman and Ramseur, 1979; Holland and Villarreal, 1975; Holland *et al.*, 1980; Jacobson and Pfau, 1980; Rima and Martin, 1976).

The coronavirus group has a positive single-stranded RNA genome with a molecular weight (MW) of  $5.4-6.9 \times 10^6$  (Siddell *et al.*, 1982). So far, there have been no reports on the existence of coronavirus

DI particles. The JHM strain of mouse hepatitis virus (MHV), a coronavirus (Bailey *et al.*, 1949; Cheever *et al.*, 1949; Robb and Bond, 1979; Siddell *et al.*, 1982), has now been examined for the generation of DI particles. We have observed marked reduction of infectivity in the course of serial undiluted passages of JHM virus in DBT cell culture. The progeny viruses interfered with the replication of the original JHM virus but not with vesicular stomatitis virus. This interference was mediated by particles of JHM virus which possessed the properties of DI particles cited above.

### MATERIALS AND METHODS

#### *Cell Culture*

DBT cells (Hirano *et al.*, 1974; Kumanishi, 1967) were used for propagation of viruses and infectivity assays. They were grown in Eagle's minimal essential medium (MEM, Nissui, Tokyo) containing 10% calf serum and 10% tryptose phosphate

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broth (Difco) (GM). The concentration of calf serum was reduced to 5% in the maintenance medium (MM) used for virus harvesting.

#### *Viruses and Infectivity Assay*

To avoid contamination of the standard virus stock with DI particles, plaque purified MHV (JHM strain) (Hirano *et al.*, 1981; Makino *et al.*, 1983) was propagated on DBT cells at a multiplicity of infection (m.o.i.) of 0.0002 and at 15 hr postinfection (p.i.) culture fluid was harvested and clarified by low speed centrifugation. This virus was then propagated twice on DBT cells at an m.o.i. of 0.01. At 16 hr p.i. the culture fluid was harvested, clarified, and then stored at  $-70^{\circ}$ . The infectivity of this standard JHM virus was  $2.0 \times 10^6$  plaque-forming units (PFU)/0.2 ml. Vesicular stomatitis virus (VSV), strain New Jersey, was propagated on DBT cells and stored at  $-70^{\circ}$ . Infectivities of JHM virus and VSV were determined by the plaque assay system described previously (Hirano *et al.*, 1974; Makino *et al.*, 1982).

#### *Serial Undiluted Passages*

DBT cell monolayers ( $2 \times 10^6$  cells) in 35-mm plastic dishes (Corning, New York) were washed once with MEM and inoculated with 0.2 ml of the standard JHM virus at an m.o.i. of 1.0. After adsorption for 60 min at  $37^{\circ}$ , unabsorbed virus was removed and the cells were washed once with MEM and cultured with 2 ml of MM/dish at  $37^{\circ}$ . The culture fluids were harvested at 14 hr p.i. and clarified, and the supernatants were stored at  $-70^{\circ}$ . Two hundred microliters of undiluted samples of passage 1 was used to infect a fresh DBT culture, and each successive passage was carried out in the same way.

#### *Preparation of the Standard JHM Virus for Interference Assay*

JHM virus was propagated on DBT cells after infection at an m.o.i. of 1.0 and was harvested at 14 hr p.i. The culture fluid was clarified by centrifugation at 8000 rpm for 30 min. One liter of the supernatant

was subjected to salt precipitation with ammonium sulfate (50% saturation) followed by centrifugation at 8000 rpm for 30 min. The pellet was resuspended in NTE buffer (containing 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.2, and 0.001 M ethylenediamine-tetraacetic acid (EDTA)) and the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was then placed on a discontinuous sucrose gradient consisting of 60, 30, and 20% (w/v) sucrose (3:2:2 in volume, respectively) and centrifuged at 27,000 rpm for 3 hr at  $4^{\circ}$  in a Beckman SW 28.1 rotor. A virus band at the interface between 60 and 30% sucrose was collected and diluted sixfold with GM. This diluted virus stock contained  $2.0 \times 10^7$  PFU/0.2 ml and was stored at  $-70^{\circ}$ . This partially purified JHM virus was used as the standard JHM virus for interference assay.

#### *Interference Assay*

DBT cell monolayers ( $3 \times 10^5$  cells) prepared in 24-well multiwell plates (Coster, Cambridge) were washed once with MEM, and inoculated with the mixture of 50  $\mu$ l of the standard JHM virus or VSV and 100  $\mu$ l of test virus samples. A mixture of 50  $\mu$ l of the standard JHM virus or VSV and 100  $\mu$ l of MM was inoculated as a control. After 60 min at  $37^{\circ}$ , unabsorbed viruses were removed and the cells were washed once with MEM and incubated at  $37^{\circ}$  for 13 hr with 1 ml of MM/well.

#### *Radiolabeling and Purification of Virus*

DBT cells grown in 60- or 35-mm plastic dishes were inoculated with 0.4 and 0.2 ml of virus samples, respectively. After adsorption the inoculum was removed and the cells were labeled with [ $^3$ H]uridine or [ $^3$ H]leucine according to methods described previously (Makino *et al.*, 1983). For the preparation of  $^{32}$ P-labeled virus RNA, cultures were incubated for 2 hr before virus inoculation with MEM in which the concentration of phosphate was reduced to 1/10. After virus adsorption, the inoculum was replaced by phosphate-free MEM containing 10% dialyzed calf serum and 200  $\mu$ Ci of  $^{32}$ P (Japan Radioisotope Association,

Tokyo) per milliliter. Radiolabeled virus was collected after 14 hr of infection and purified from the medium of the infected culture as described previously (Makino *et al.*, 1983).

#### *SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

The discontinuous SDS-polyacrylamide system of Laemmli (1970) was used as described previously (Makino *et al.*, 1983). After electrophoresis, gels were further processed for fluorography using sodium salicylate (Chamberlain, 1979).

#### *Preparation of Virion RNA*

Pellets of purified viruses were suspended in NTE buffer containing 0.5% SDS and 100  $\mu\text{g}/\text{ml}$  of proteinase K (Boehringer-Mannheim, West Germany). The suspension was incubated at 50° for 5 min, at 25° for 25 min, and then RNA was extracted with phenol-chloroform-isoamyl alcohol (50:50:1). The final aqueous phase was made 0.25 *M* in sodium chloride and the RNA was precipitated at -20° after the addition of 2.5 vol of ethanol. After centrifugation and one washing with 75% ethanol, RNA was dried and dissolved in a small volume of redistilled water.

#### *Agarose Gel Electrophoresis*

1. *Analytical electrophoresis.* Following denaturation of the RNA with glyoxal treatment, agarose gel electrophoresis was performed as previously described (McMaster and Carmichael, 1977). After electrophoresis the gels were washed with water and dried under vacuum, and then exposed to a Kodak XS-1 film with an intensifying screen.

2. *Preparative electrophoresis.* Radiolabeled RNA was dissolved in 10 *mM* sodium phosphate, pH 7.0, containing 1 *mM* EDTA, 0.1% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol FF and mixed with equal volume of glycerol. The solution was heated for 5 min at 56°, rapidly cooled on ice, and placed in a slot of a horizontal 1% agarose in electrophoresis buffer (40 *mM* Tris, 20 *mM* sodium acetate, 33 *mM* acetic

acid, 2 *mM* EDTA, pH 7.4) supplemented with 6 *M* deionized urea. Electrophoresis was performed at 4°. RNA bands were located by autoradiograph of the wet gel, excised, and kept at -20°. The gel slices were melted for 10 min at 65° and 20  $\mu\text{g}$  of yeast tRNA (type x; Sigma) was added to each sample. Then the RNA was eluted from gel slices by the methods of Langridge *et al.* (1980).

#### *Oligonucleotide Fingerprinting*

The purified <sup>32</sup>P-labeled RNA was exhaustively digested with RNase T<sub>1</sub> (Sankyo, Tokyo) and analyzed by two-dimensional polyacrylamide gel electrophoresis by a modification of the procedure of de Wachter and Fiers (1972). The first dimension was performed on 7.2% polyacrylamide-0.09% bisacrylamide gel slabs at pH 3.3 containing 6 *M* urea. Electrophoresis was carried out at 1000 V for 4 to 5 hr. The second dimension was performed on 22% polyacrylamide-1.54% bisacrylamide gel slabs at pH 8.2 and 650 V for 16 to 19 hr. After electrophoresis, the gel was wrapped with Saran Wrap and exposed to Kodak XAR5 or XS-1 film with an intensifying screen at 4° for an appropriate length of time.

## RESULTS

The culture fluid from 25 undiluted passages was assayed for infectivity at each passage (Fig. 1). From passages 1 to 5, a constant level of infectious viruses was produced. During passages 6 to 8, however, the yield of infectious viruses was significantly reduced. At passage 9, infectivity of the culture supernatant increased, but it was again reduced from passages 10 to 12. These cyclic increases and decreases in infectivity were observed until passage 25. Severe syncytial formation was observed in all 25 passages.

To determine if the reduction in the yield of infectious virus was due to the presence of DI particles, an interference analysis was performed with several culture fluid samples at different passage levels. As shown in Table 1, the multiplication of the standard JHM virus was specifically in-

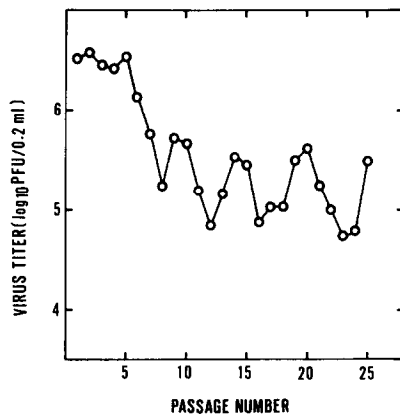


FIG. 1. Fluctuation of infectivity during serial passages without dilution of JHM virus in DBT cells. Serial undiluted passages of JHM virus in DBT cells were carried out as described under Materials and Methods.

hibited by passaged virus stock samples at passage levels 10, 15, and 20. A slight inhibition was observed with a sample at passage 5. However, the growth of VSV was not interfered with by passages 10 and 20 virus samples.

To test whether this interference was mediated by DI particles or another agent, such as interferon, passage 1 and 10 viruses were first metabolically labeled with [<sup>3</sup>H]uridine and centrifuged to equilibrium. Infected culture fluid (400 ml) from passages 1 and 10 was then precipitated with ammonium sulfate, as described under Materials and Methods, and this unlabeled material was mixed with the partially purified labeled virus from the corresponding passage level. These mixtures were then applied to a 20 to 60% continuous sucrose gradient. As shown in Fig. 2, a single radioactive peak, corresponding to the infectivity peak, appeared in both virus preparations at a buoyant density of 1.196 g/cm<sup>3</sup>. The activity of interference with standard JHM virus was detected in the virus preparation of passage 10 but not in that of passage 1. This interference activity coincided with the peak of radioactivity and infectivity. The results indicated that the agent causing interference could not be separated from standard infectious virions by this sucrose equilibrium centrifugation.

TABLE 1  
INTERFERING ACTIVITY OF PASSAGE VIRUS<sup>a</sup>

Infection		Test virus (passage)		PFU/0.2 ml at 14 hr p.i.	% yield
Standard virus	m.o.i.	m.o.i.	m.o.i.		
JHM virus	5	—	—	$1.5 \times 10^{6b}$	100
	5	5	5	$1.3 \times 10^6$	87
	5	10	0.8	$6.4 \times 10^5$	43
	5	15	0.5	$8.3 \times 10^5$	55
	5	20	0.7	$7.3 \times 10^5$	49
VSV	10	—	—	$4.2 \times 10^{7c}$	100
	10	JHM virus	5	$4.4 \times 10^7$	>100
	10	10	0.8	$4.5 \times 10^7$	>100
	10	20	0.7	$4.2 \times 10^7$	100

<sup>a</sup> DBT cells ( $3 \times 10^5$  cells) in 24-well plates were infected with 50  $\mu$ l of standard JHM or VSV and 100  $\mu$ l of test samples. After 60 min at 37° inoculum was removed and cells were washed once with MEM. The cells were cultured with 1 ml of MM for 13 hr at 37° and medium was harvested, centrifuged, and assayed for infectivity.

<sup>b</sup> JHM virus titer.

<sup>c</sup> VSV titer.

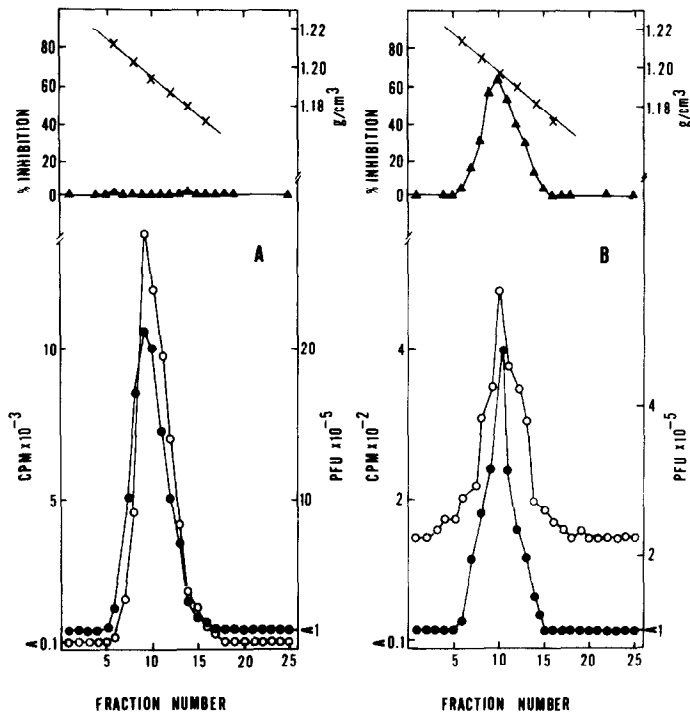


FIG. 2. Sucrose equilibrium centrifugation of passages 1 and 10 viruses. DBT cells ( $5 \times 10^6$  cells) in 60-mm dishes were infected with either the standard JHM virus (A) or passage 9 virus (B). Virus was labeled for 13 hr p.i. with [ $^3\text{H}$ ]uridine (100  $\mu\text{Ci}/\text{ml}$ ) and partially purified by a discontinuous sucrose gradient (60, 30, and 20% sucrose). Then the partially purified virus was mixed with unlabeled virus of the same passage level, prepared by precipitation with ammonium sulfate, and then this mixture was applied to a 20 to 60% continuous sucrose gradient and centrifuged at 27,000 rpm for 18 hr at 4° by Beckman SW 28.1 rotor. Then the gradient was fractionated into 25  $\times$  0.35 ml portions. Portions of each fraction were analyzed for radioactivity (○) and PFU (●). The interference activity (▲) was measured by the use of 1:6 dilution of the sample with GM and selected fractions were also examined for density (×).

In the next series of experiments viruses of passages 1 and 10 were metabolically labeled with [ $^3\text{H}$ ]leucine, purified, and subjected to SDS-PAGE analysis (Fig. 3). Viruses of passages 1 and 10, both showed four major polypeptides with MW of 260K, 105K, 60K, and 23K. A 21K polypeptide was also sometimes found, but in variable amounts. The only obvious difference between the structural polypeptides of viruses from passages 1 and 10 was a 65K protein, which was present in much larger amounts in passage 10 virus. This protein was also present in passage 1 virus but to obtain clear identification it was necessary to apply a greater amount of radioactivity to the gel (lane c).

Virion RNAs labeled with  $^{32}\text{P}$  were extracted from purified virus samples at passage levels of 1, 3, 4, 5, 10, 15, and 20. The RNAs were denatured with glyoxal and subjected to agarose gel electrophoresis. Virus RNAs from passages 1, 3, and 4 showed a single band with a MW of  $5.4 \times 10^6$ , which was identical to the MW of MHV genome RNA described previously by others (Fig. 4) (Lai *et al.*, 1981; Lai and Stohman, 1978). On the other hand, virion RNAs from passages 5, 10, 15, and 20 showed two bands, one identical to that from passages 1 to 4 and a second having a lower MW of  $5.2 \times 10^6$ . To confirm that this lower MW RNA species was a defective RNA and not due to conformational

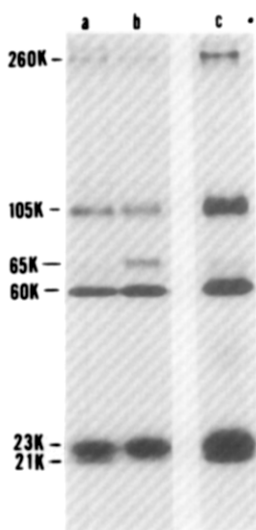


FIG. 3. Structural proteins of passage 1 and 10 viruses. Viruses were labeled with [ $^3\text{H}$ ]leucine and purified as described under Materials and Methods. Fractions from 5 to 15 in Fig. 2 were pooled, diluted, and pelleted at 45,000 rpm for 60 min at 4° in a Beckman SW 50.1 rotor. The pelleted viruses were treated with sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue), and boiled for 2 min. Electrophoresis was performed on a 7.5 to 15% polyacrylamide gel. The ratio of radioactively labeled virus applied was a:b:c = 1:1:2.5; lane a, passage 1 virus; lane b, passage 10 virus; lane c, passage 1 virus.

changes of the genomic RNA, the RNAs with MW of  $5.4 \times 10^6$  (genomic RNA) of passages 1 and 10 viruses and the lower MW RNA species of passage 10 viruses were compared by oligonucleotide fingerprinting (Fig. 5). Poly(A) tracts are seen as streaks in the lower left-hand corners of every fingerprint indicating that all RNA species were polyadenylated. The oligonucleotide fingerprinting pattern of genomic RNA of passage 1 virus is almost identical to that reported by others. The numbering of oligonucleotides in this paper is therefore as described previously (Lai *et al.*, 1981; Stohlman *et al.*, 1982). However, three oligonucleotides (numbered as 2a, 2b, and 42a) appear not to be present in the previously reported fingerprints. Oligonucleotide fingerprinting of genomic RNA of passage 10 was essentially identical to that of passage 1 virus. However, an oli-

gonucleotide (42b) (indicated by an arrow) appeared in the genomic RNA of passage 10 and an oligonucleotide, 42a, had disappeared (indicated as an open circle). The oligonucleotide fingerprinting of the lower MW RNA species of passage 10 virus showed the loss of nine oligonucleotides including 42a and 42b (indicated as open circles) and the appearance of three additional oligonucleotides (indicated by arrows). These studies clearly indicated that the lower MW RNA species had undergone deletions. Thus, these data strongly suggested that DI particles were present in culture fluids after passage 5.

For further confirmation of the presence of DI particles, high dilution serial passages of virus stock containing DI particles were conducted. DBT cells were first infected with passage 10 virus samples at an m.o.i. of 0.0002, and the virus yield was subsequently inoculated onto DBT cells at an m.o.i. of 0.1. This virus stock after passages with dilution (SPD) was shown to possess an infectivity of  $1.5 \times 10^6$  PFU/0.2 ml and did not interfere with the multiplication of the standard JHM virus (data

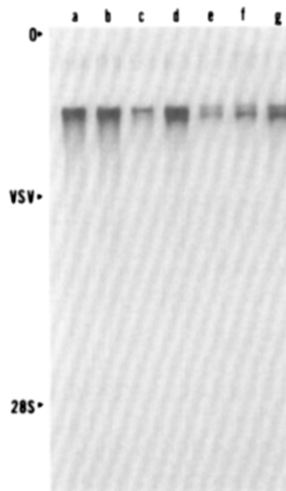


FIG. 4. Agarose gel electrophoresis of glyoxal denatured genomic RNAs from different passage levels.  $^{32}\text{P}$ -labeled RNAs from purified virions (i.e., fractions from 5 to 15 in Fig. 2) were prepared and analyzed as described under Materials and Methods. O: origin. Passage levels: a = 1, b = 3, c = 4, d = 5, e = 10, f = 15, g = 20. VSV and 28S indicate the localization of VSV RNA and 28S rRNA.

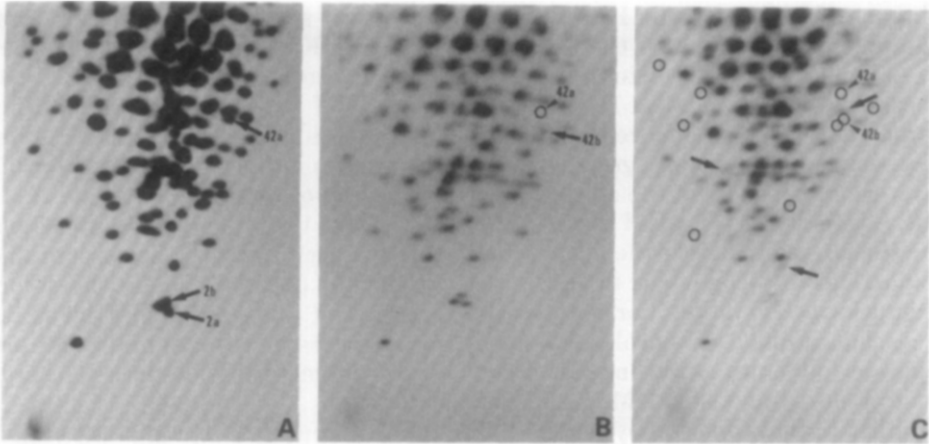


FIG. 5. Oligonucleotide fingerprinting of genomic RNA of passage 1 virus (A), genomic RNA of passage 10 virus (B), and the lower MW RNA of passage 10 virus (C). The first-dimension electrophoresis is from left to right. The second dimension is from bottom to top. The significance of the numbers, open circles, and arrows is given in the text.

not shown). Viral RNA extracted from the SPD contained a single RNA with a MW of  $5.4 \times 10^6$  (Fig. 6) being identical to that of the virus passage 1.

These data confirmed that the smaller size RNA was DI RNA and that DI particles were eliminated by serial passages with dilution.

#### DISCUSSION

DI particles are deletion mutants which cannot replicate by themselves but interfere specifically with replication of the homologous virus, which is itself required for the generation and replication of the DI particles. After serial passages without dilution, DI particles were generated in our virus stock of JHM virus. These particles were characterized by (1) interference with the multiplication of homologous JHM virus but not with that of VSV, (2) being inseparable from infectious JHM virions by sucrose equilibrium centrifugation, (3) having structural proteins almost identical to the original JHM virus, (4) having a smaller size RNA (MW:  $5.2 \times 10^6$ ) that had lost several RNase T<sub>1</sub> resistant oligonucleotides, (5) being excluded readily from the virus stock by serial passages with dilution.

We and others have also searched for the presence of DI particles in MHV per-

sistent infection *in vitro* (Hirano *et al.*, 1981; Robb and Bond, 1979; Stohlman and Weiner, 1978) and the generation of DI particles by serial passages without dilution has been attempted by several laboratories (Leibowitz *et al.*, 1981; Robb and Bond, 1979;



FIG. 6. Agarose gel electrophoresis of glyoxal denatured genomic RNA from passages with dilution. a = Diluted passage virus RNA from passage 10 (SPD), b = RNA of passage 15 virus.



Stohlman and Weiner, 1978). However, all these studies failed to detect the presence of DI particles of MHV. Stohlman and Weiner (1978) failed to detect DI particles using murine neuroblastoma cells and DBT cells by serial undiluted passages of viruses which were released from the culture persistently infected with JHM virus. The reason for failure to detect the generation of DI particles of JHM virus in these experiments is unclear, but minor genetic differences might play some roles, because although the oligonucleotide fingerprinting pattern of our standard JHM virus was very similar to that reported by others (Lai and Stohlman, 1981), some differences existed (see Fig. 5).

During undiluted passages DI RNA was first detected at passage 5, and the virus yield was gradually reduced thereafter until passage 8, indicating that DI particles, generated at passages 4 to 5, accumulated in the culture and played a crucial role in the reduction of the infectivity. After reduction of the yield of infectious virus, fluctuating patterns of infectivity were observed. Such a phenomenon has been described in other viruses during passages without dilution (Holland *et al.*, 1980; Huang and Baltimore, 1977), indicating that the DI particles can replicate only with the aid of helper standard infectious virus and that the replication of the helper standard virus was inhibited by DI particles. The fluctuating pattern of infectivity has also been observed in the case of persistent infections with many viruses in tissue culture, in which DI particles have been detected.

The DI particles of JHM virus were not separable from infectious JHM particles by sucrose centrifugation. This was presumably due to the relatively small deletions present in the RNA of DI particles and there being no major difference between the structural proteins of these particles and standard JHM virions. However, Bruton and Kennedy (1976) reported that Semliki Forest virus DI particles could be separated from the standard virus using CsCl but not sucrose centrifugation. So the purification and separation of DI particles of JHM virus might be possible using these techniques.

In our studies, four major proteins (260K, 105K, 60K, and 23K) and minor 21K and 65K proteins were identified at passage 1 of JHM virus. As already described (Makino *et al.*, 1983) the 260K protein in our studies is the counterpart of gp170 described by others (Siddell *et al.*, 1981; Siddell *et al.*, 1982). The proteins of passage 10 virus, rich in DI particles, contained no significant difference from the structural proteins of passage 1 JHM virus, except for an increased amount of the 65K protein. At the moment this observation cannot be explained.

The mapping of T<sub>1</sub> resistant oligonucleotides on the genome of JHM virus has been reported by Stohlman *et al.* (1982), and therefore it should be possible to localize the deletion site(s) in the RNA of the DI particles reported here. Comparing the oligonucleotide fingerprinting of DI RNA with standard virus RNA, it seems that the deletion(s) were located within a region 2 to 7 kilobases (kb) from the 3' end of the genome. It is not possible to conclude, however, whether there is only a single, or multiple deletion sites. More detailed mapping of the oligonucleotides in this region of the genome would presumably define the precise location of the deletion site(s).

The mechanisms of interference and generation of MHV DI particles are not understood. Studies on the detail gene structure of MHV DI RNA described in this paper are now in progress and may help to elucidate these questions.

#### ACKNOWLEDGMENTS

We thank Dr. S. Siddell and Dr. M. Skinner for valuable discussion and correction of the manuscript, and Dr. M. Hayami and Dr. Y. Yoshikawa for their suggestions and critical comments.

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