J. gen. Virol. (1982), 63, 265–275. Printed in Great Britain



Key words: MHV/coronavirus/plaque variants/oligonucleotide maps

# Murine Coronaviruses: Isolation and Characterization of Two Plaque Morphology Variants of the JHM Neurotropic Strain

By STEPHEN A. STOHLMAN,* PETER R. BRAYTON, JOHN O. FLEMING, LESLIE P. WEINER AND MICHAEL M. C. LAI

*Departments of Neurology and Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033, U.S.A.*

(*Accepted 21 June 1982*)

SUMMARY

Two plaque-size variants of the neurotropic JHM strain of mouse hepatitis virus have been isolated from the virus stock after eight serial passages in suckling mouse brain. One variant, JHM-DL, produces large plaques, while the other, JHM-DS, produces small plaques in tissue culture. DS replicates more slowly, has a lower virus yield *in vitro*, and is less virulent for mice than DL. They also differ in their pathogenicity for mice: JHM-DL infection results in acute encephalomyelitis while JHM-DS infection results in demyelination. Oligonucleotide fingerprint analysis of the RNA genomes of these two variants revealed that they had almost identical genetic sequences. Each variant, however, had a unique oligonucleotide spot not found in the other. The unique spot of the large plaque variant, JHM-DL, was localized at approximately 3 to 5 kb from the 3′ end, while the JHM-DS unique spot was mapped at 14 to 15 kb from the 3′ end of the genome. We have further shown that these oligonucleotide changes are not correlated with the plaque morphology. These two viruses may be useful for studying the molecular basis of virus-induced demyelination.

## INTRODUCTION

Murine coronaviruses, or mouse hepatitis viruses (MHV), have been isolated from various mouse colonies for the last 30 years. Generally, the isolation has been from animals dying of acute hepatitis (Dick *et al.*, 1956; Gledhill & Niven, 1955; Manaker *et al.*, 1961); however, isolates have also been obtained from mice with hind leg paralysis due to demyelination in the central nervous system (Cheever *et al.*, 1949), from mice with acute enteritis (Hierholzer *et al.*, 1979; Ishida *et al.*, 1978; Sugiyama & Amano, 1980), and from nude mice with wasting disease (Hirano *et al.*, 1975). In general, these viruses establish latent gastrointestinal infections and are transmitted horizontally (Gledhill & Niven, 1955). Stress of latently infected mice or experimental inoculation of these viruses into mice usually results in acute hepatitis.

We have been interested in a particular strain of MHV, the JHM strain (MHV-JHM), which was the first reported isolate of MHV and was recovered from a mouse with hindleg paralysis (Cheever *et al.*, 1949). Experimentally, MHV-JHM differs from the other strains of MHV in that it induces a generalized encephalomyelitis with necrosis of both grey and white matter (Cheever *et al.*, 1949; Nagashima *et al.*, 1978; Weiner, 1973). It also differs from most other virus infections of the central nervous system in that it specifically infects oligodendroglia during the acute disease. This infection results in selective loss of myelin while sparing axons. MHV-JHM also produces a persistent oligodendroglial infection which results in a progressive chronic demyelinating process (Herndon *et al.*, 1975; Stohlman & Weiner, 1981). Thus, this virus provides an interesting system for the study of the molecular basis of virus-induced demyelination. For this purpose, virus mutants of the MHV-JHM strain affecting its neurotropic pathogenicity will be very useful. As an initial approach toward the goal of understanding the molecular basis of neuropathogenicity, we have isolated two MHV-JHM variants with altered plaque morphology. These two strains were found to have altered virulence

0022-1317/82/0000-5101 $02.00 

for mice and altered neurotropic pathogenicity. In this report, we describe the biological characteristics and the sequence divergence of these two variants.

Coronaviruses contain a single- and positive-stranded 60S RNA genome (Lai & Stohlman, 1978). This RNA, with a mol. wt. of $5{\cdot}4 \times 10^6$, is enough to code for all of the known virus structural proteins of MHV and, in addition, several non-structural proteins. A tentative genetic map of the genes coding for some of these proteins has been proposed (Lai *et al.*, 1981). In this paper, we localized the detectable divergence on the RNA genome in the genetic sequences of these MHV-JHM plaque morphology variants. These data will be useful for the study of the biological functions of the gene products of MHV.

## METHODS

*Viruses and cells.* The two virus variants compared in this study were obtained from a pool of MHV-JHM passaged eight consecutive times by intracerebral (i.c.) inoculation in suckling mice (Weiner, 1973). This suckling mouse brain (SMB) pool induces acute encephalomyelitis with very little demyelination following i.c. inoculation into mice. Plaque assay of SMB passage 8 (SMBp8) on L929 and DBT cells resulted in a heterogeneous population of plaque sizes. Large and small plaques were picked and further purified by sequential plaque purification on monolayers of DBT cells as previously described (Stohlman & Weiner, 1978; Stohlman *et al.*, 1979). The large plaque variant, JHM-DL (DL), was plaque-purified four times, and serially passaged in DBT cells to prepare virus stocks. The DL used in these studies was from the 4th and 5th *in vitro* passages subsequent to the final plaque isolation. These passages retained the homogeneous large plaque morphology of the parent cloned virus, designated DL passage zero. The small plaque variant, JHM-DS (DS), was cloned by six consecutive plaque purifications on DBT cells. The DS used in these studies was from the 6th serial passage subsequent to plaque purification on DBT cells. This passage retained the homogeneous small plaque morphology of the parent cloned virus, designated DS passage zero. L2 and 17 clone 1 (17 CL-1) cells were obtained from Dr L. Sturman (Albany, N.Y., U.S.A.). Plaque sizes were determined by assay on DBT cells after 48 h incubation as previously described (Stohlman & Weiner, 1978). Briefly, the infected cell monolayers were fixed with 10% formalin, stained with crystal violet, and the plaque sizes measured using a calibrated ocular.

*Growth and radiolabelling of virus.* Viruses were propagated on 15-cm dishes containing monolayers of DBT cells, originally derived from a murine astrocytoma (Hirano *et al.*, 1975). Virus was adsorbed to the cells at a multiplicity of 0·1 to 1·0 for DL and 0·01 to 0·05 for DS at 37 °C for 1 h. Following adsorption, the inoculum was removed and 25 ml of Dulbecco's modified minimal essential medium (DMEM) containing 1% heat-inactivated (56 °C for 30 min) foetal calf serum (FCS) was added. After 4 h for DL and 10 h for DS, the medium was removed and replaced with phosphate-free DMEM containing 1% dialysed FCS and 200 μCi/ml [$^{32}$P]orthophosphate (ICN Pharmaceuticals). Supernatants were collected 8 h later.

For determination of the kinetics of virus growth, DBT cells were infected with DL or DS at an identical multiplicity of 0·03. After virus adsorption at 37 °C for 1 h, the inoculum was removed and DMEM containing 1% FCS was added. Virus titres in the media were determined by plaque assays on DBT cells as described previously (Stohlman & Weiner, 1978).

*Purification of virus.* Supernatants obtained from the infected cultures 8 h after the addition of radiolabel were cleared of cell debris at 15000 *g* for 30 min at 4 °C. The virus was then pelleted from the supernatants through 20% sucrose prepared in TEM buffer (0·05 M-Tris–maleate, 0·1 M-NaCl, 0·01 M-EDTA, pH6·2) at 25000 *g* in an SW27 rotor for 2·5 h at 4 °C. The pellets were resuspended in TEM and centrifuged at 40000 *g* in an SW41 rotor for 2 h in a linear 20 to 42% sucrose gradient prepared in TEM. Fractions were collected from the bottom of the centrifuge tube and those containing the peak of radioactivity (at density approximately 1·18 g/ml) were pooled.

*Extraction of virus RNA.* Virus RNA was extracted from the purified virus by a modification of published procedures (Lai & Stohlman, 1978). The virus was incubated with proteinase K (50 μg/ml) in the presence of 0·5% SDS at 37 °C for 20 min. The RNA was then extracted once with phenol/choloroform (1 : 1) and then with phenol alone. The RNA was precipitated with 3 vol. ethanol at − 20 °C overnight, pelleted by centrifugation at 20000 *g* for 15 min and then sedimented in 10 to 25% sucrose gradients made up in NTE buffer (0·01 M-Tris–HCl pH 7·4, 0·1 M-NaCl, 0·001 M-EDTA) in an SW50.1 rotor at 50000 *g* for 60 min. The 60S RNA was pooled and precipitated with 3 vols ethanol.

*Oligonucleotide fingerprinting.* The $^{32}$P-labelled 60S RNA was exhaustively digested with RNase $T_1$ and analysed by two-dimensional polyacrylamide gel electrophoresis according to a modification (Lai & Stohlman, 1981; Lai *et al.*, 1981) of the procedure of De Wachter & Fiers (1972). Briefly, the first separation was performed on 10% polyacrylamide gel slabs (30 × 10 × 0·15 cm) at pH 3·3, 700 V for 4 h. The second separation was performed on 22% polyacrylamide gel slabs (40 × 35 × 0·075 cm) at pH 8·0, 650 V for 16 h. After electrophoresis, the gel was wrapped with cellophane and exposed to Kodak BB-5 films, with an intensifying screen, at 4 °C for appropriate lengths of time.

*Fractionation of poly(A)-containing RNA for oligonucleotide mapping.* The $^{32}$P-labelled 60S RNA was fractionated for oligonucleotide mapping according to published procedures (Lai *et al.*, 1981). Briefly, the RNA was incubated with 0·05 M-sodium carbonate at 50 °C for 1 min, and then neutralized with acetic acid and precipitated with 2·5 vol. ethanol. The RNA was pelleted by sedimentation at 20000 *g* for 15 min and then fractionated by oligo(dT)–cellulose column chromatography. In the latter part of these studies, the alkali digestion of the RNA was omitted. Instead, the total RNA, without being subjected to sucrose gradient sedimentation, was used directly for oligo(dT)–cellulose chromatography. The RNA prepared by this procedure contained enough nicks for oligonucleotide mapping.

The poly(A)-containing RNA was sedimented through a 10 to 25% sucrose gradient prepared in a buffer containing 0·01 M-Tris–HCl pH 7·4, 0·01 M-NaCl and 0·05% SDS in an SW41 rotor at 40000 *g* at 20 °C for 4·5 h. The RNA fractions of different sedimentation values were pooled separately, and used for $T_1$ oligonucleotide fingerprinting.

## RESULTS

### *Isolation and biological characteristics of plaque-size variants of MHV-JHM*

Virus pools prepared from the 6th and 7th serial passages (SMBp6 and SMBp7) of MHV-JHM in suckling mouse brain at limiting dilution both produce primary demyelination following intracranial inoculation into mice (Weiner, 1973). Plaque assay of these two pools shows uniform-sized plaques on L2, L929 and DBT cells. The 8th serial passage of MHV-JHM (SMBp8) was prepared from the 7th-passage virus by inoculating approximately 100 p.f.u. per suckling mouse. Unexpectedly, this virus pool produced acute encephalomyelitis in young adult mice with little detectable demyelination (L. P. Weiner, unpublished data). SMBp8 also differed from earlier passages in that it produced a wide spectrum of plaque sizes when assayed on L929 or DBT cells. To isolate possible genetic variants of JHM, we selected the largest (JHM-DL) and the smallest (JHM-DS) plaques of SMBp8, which were then serially plaque-purified on DBT cells. The cloned DL produced plaques of 1·18 ± 0·24 mm in diameter while DS produced plaques with a diameter of only 0·48 ± 0·13 mm on DBT cells when measured 48 h after infection. This contrasted with SMBp7 which produced uniform plaques of 1·02 ± 0·22 mm in diameter when assayed under the same conditions. Furthermore, the DL and DS variants were assayed on several other cell lines, including L929, L2 and 17 CL-1, and produced large and small plaques respectively, compared to those produced by SMBp7 (data not shown). These data suggested that the difference in their plaque size was determined by the genetic information of the virus, rather than by host cell factors. DL and DS were serially passaged 11 and 15 times respectively on DBT cells without change in plaque size from that of the cloned parents, indicating that these two JHM variants are genetically stable.

The DL and DS variants assayed at different temperatures were found to form plaques with equal efficiency at 32, 37 and 39 °C (Table 1). This property is similar to that of SMBp7, as previously reported (Stohlman & Weiner, 1978; Stohlman *et al.*, 1979). It is noteworthy that the titre of DS is about 30- to 50-fold lower than that of DL at all of the temperatures in the assay.

The small size of the plaques produced by the DS variant suggested that it might have a slower growth rate than the DL variant. We therefore compared the kinetics of virus growth of DL and DS on DBT cells at identical multiplicities. Fig. 1 shows that the DL variant was released into

Table 1. *Titration of the DL and DS variants of MHV-JHM at different temperatures**

| Temperature (°C) | Titre (p.f.u./ml) | |
|---|---|---|
| | DL | DS |
| 32 | $2{\cdot}4 \times 10^6$ | $4{\cdot}7 \times 10^4$ |
| 37 | $3{\cdot}1 \times 10^6$ | $9{\cdot}0 \times 10^4$ |
| 39 | $8{\cdot}4 \times 10^6$ | $1{\cdot}8 \times 10^5$ |

* The infected DBT cells were incubated at different temperatures for 72 h and plaques visualized with neutral red.

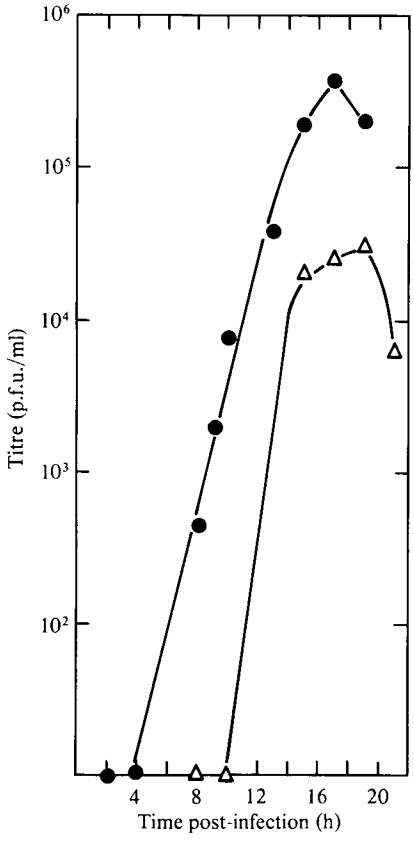


Fig. 1

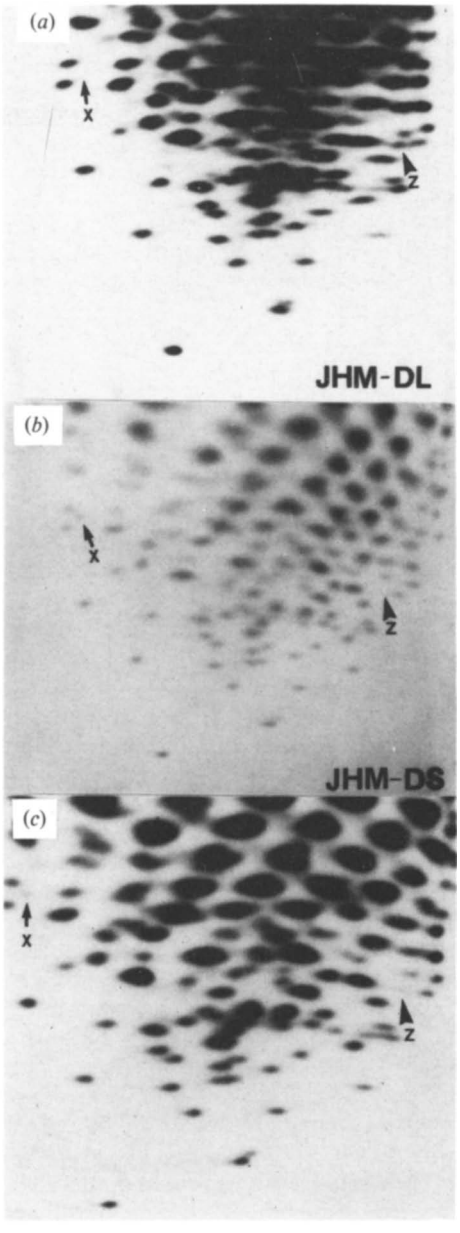


Fig. 2

Fig. 1. The kinetics of growth of JHM-DL and JHM-DS in DBT cells. The DBT cells were infected with JHM-DL (●) or JHM-DS (△) at a multiplicity of 0·03. An aliquot of the media from the infected cells was withdrawn every 2 h and the virus titres in the supernatant were assayed on DBT cells.

Fig. 2. Oligonucleotide fingerprints of (*a*) JHM-DL, (*b*) JHM-DS and (*c*) equal mixture of DL and DS. X is the DS-specific oligonucleotide while Z is the DL-specific oligonucleotide. The first-dimension electrophoresis is from left to right. The second dimension is from bottom to top.

the supernatant well before the DS variant, although both reached maximum yield at approximately the same time. Furthermore, the virus yield of DS was consistently lower than that of DL. This result suggests that the growth rate of DS is slower.

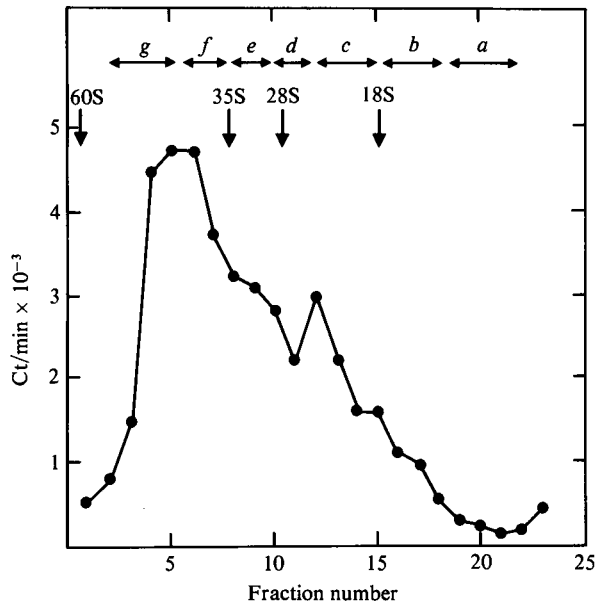


Fig. 3. Sucrose gradient sedimentation of the partially degraded poly(A)-containing JHM-DL RNA. The $^{32}$P-labelled 60S RNA of JHM-DL was briefly degraded with 0·05 M-$Na_2CO_3$ at 50 °C for 1 min and the poly(A)-tagged RNA was selected by binding to oligo(dT)–cellulose. These RNAs were then sedimented through a 10 to 25% sucrose gradient made in 0·01 M-NaCl and 0·01 M-Tris–HCl pH 7·4 in a Beckman SW41 rotor at 40000 *g* for 4·5 h. The RNA fractions of different size were pooled as indicated.

### *Virulence and pathogenicity*

Since these MHV-JHM plaque-size variants were derived from a virus pool producing encephalomyelitis and little demyelination, we initiated studies on the relative virulence and pathogenicity of DL and DS. We found that the $LD_{50}$ for 6-week-old C57BL/6 mice was 1 to 5 p.f.u. for DL and approximately 1000 p.f.u. for DS by i.c. inoculation. This result indicates that DL is much more virulent for mice than is DS. It was further found that the animals injected with DL succumbed to acute panencephalomyelitis without noticeable evidence of demyelination, with a mean time of death at the 6th day post-inoculation. DS, on the hand, produced extensive myelin destruction (S. A. Stohlman *et al.*, unpublished observation), with a mean time of death at the 8th day post-inoculation. Animals surviving 1 $LD_{50}$ of DL had no evidence of chronic demyelination, while DS survivors had demyelination and virus antigen in the CNS up to 24 months post-inoculation (S. A. Stohlman *et al.*, unpublished observation). These results suggest that DL and DS have very significant differences in their virulence and pathogenicity. The pathology of brain lesions produced by DL and DS will be published elsewhere.

### *Oligonucleotide fingerprinting of the RNA genomes of JHM-DL and JHM-DS*

To characterize the genetic difference between these two MHV-JHM variants and possibly to identify the genetic basis for the difference in their *in vitro* biological and pathogenic properties, we compared their RNA genomes by oligonucleotide fingerprinting. The $^{32}$P-labelled 60S RNAs of DL and DS were digested with RNase $T_1$, and then separated by two-dimensional polyacrylamide gel electrophoresis. As shown in Fig. 2, DL and DS have very similar oligonucleotide fingerprints, sharing almost all of the $T_1$ oligonucleotides. However, DL contains a single spot, Z, which is not present in DS. On the other hand, DS contains one spot, X, which is not present in DL. The similarity of all the other oligonucleotides of DS and DL was determined by comparing fingerprints of RNA mixtures from these two virus strains with the individual fingerprints of DL and DS. As shown in Fig. 2(*c*), all the oligonucleotide spots, except X and Z, in these two RNAs co-migrate, suggesting that, except for the one specific oligonucleotide in each strain, they share the majority of their genetic sequences.

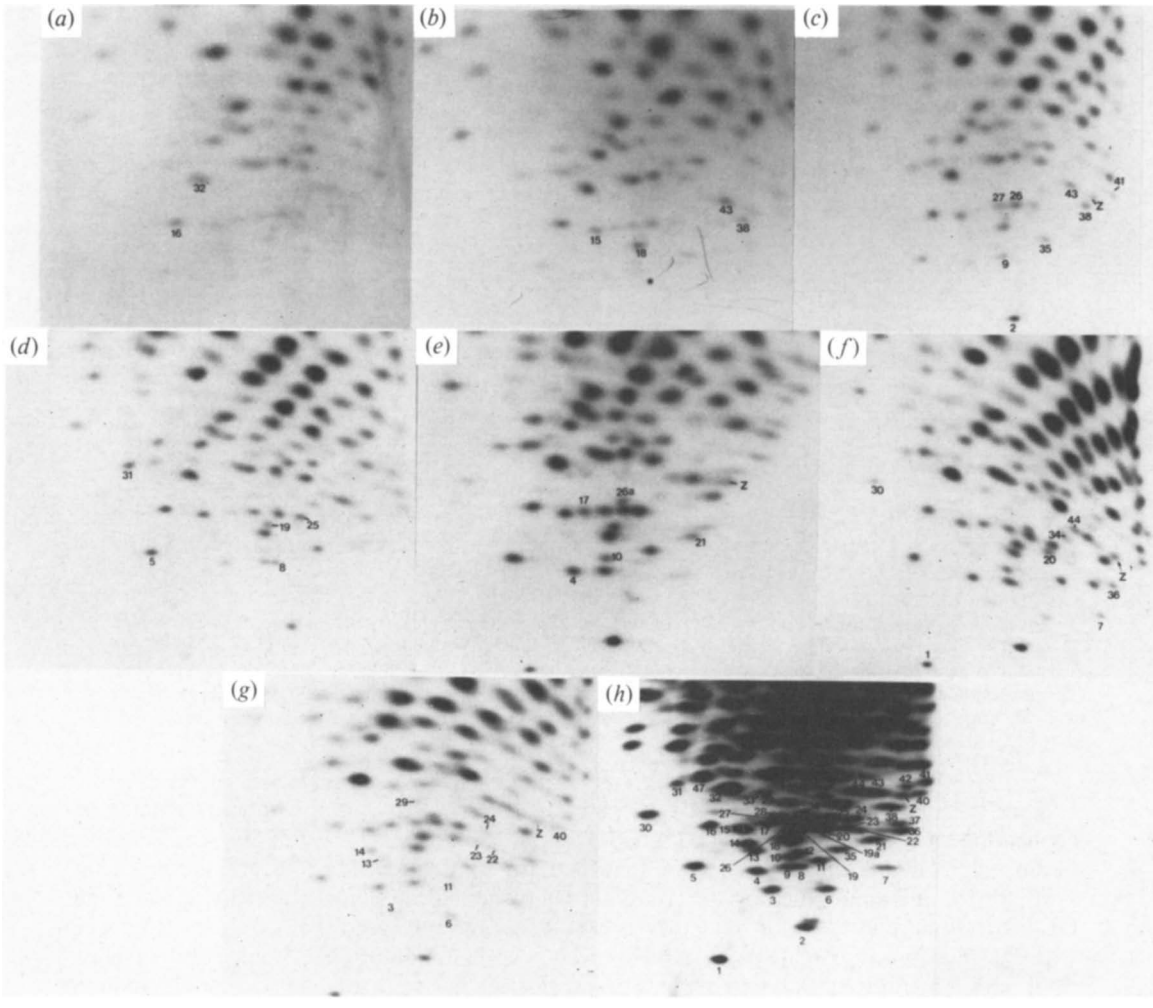


Fig. 4. Oligonucleotide fingerprints of different size classes of poly(A)-containing JHM-DL RNA. The $^{32}$P-labelled poly(A)-containing RNA of different size classes obtained from DL genomic RNA as indicated in Fig. 3(*a* to *g*) were digested with RNase $T_1$ and separated by two-dimensional polyacrylamide gel electrophoresis. The fingerprint of the 60S RNA genome was included for comparison (*h*). The numbering of oligonucleotides in (*h*) is arbitrary. The oligonucleotides which appear for the first time in the poly(A)-tagged RNA of increasing size are numbered.

### *Mapping of $T_1$ oligonucleotides on the RNA genome of JHM*

As shown in Fig. 2, the genomic sequences of DL and DS differ only in two spots on their oligonucleotide fingerprints. This finding suggests that the genetic regions corresponding to these two oligonucleotides might be the sequences responsible for the biological properties, such as plaque size, virulence and/or pathogenicity. Therefore, it was of interest to know where these genetic regions were located on the genome. To localize these sequences, partially degraded $^{32}$P-labelled RNAs from DL and DS were separately selected by chromatography on oligo(dT)–cellulose. The poly(A)-containing RNA fraction was collected and separated by sucrose gradient sedimentation. RNAs of different size were pooled separately as shown in Fig. 3 and analysed by $T_1$ oligonucleotide fingerprinting. Several of the oligonucleotide fingerprints of different-sized poly(A)-containing DL RNA fractions are presented in Fig. 4. These RNAs represent sequences starting from the 3′ end and extending for various distances toward the 5′ end of the genome. Therefore, the relative position of each oligonucleotide on the genome can be


Fig. 5. Map order of $T_1$ oligonucleotides on the RNA genome of JHM-DL and JHM-DS. The map order of the oligonucleotides was determined from Fig. 4 and Fig. 6. Since all of the spots shared between DL and DS RNAs were mapped at the same positions, they were represented only once on the map. The numbering of the oligonucleotides is as described in Fig. 4. The order of the oligonucleotides within the bracket is arbitrary. The spot Z is DL-specific while the spot X is DS-specific.

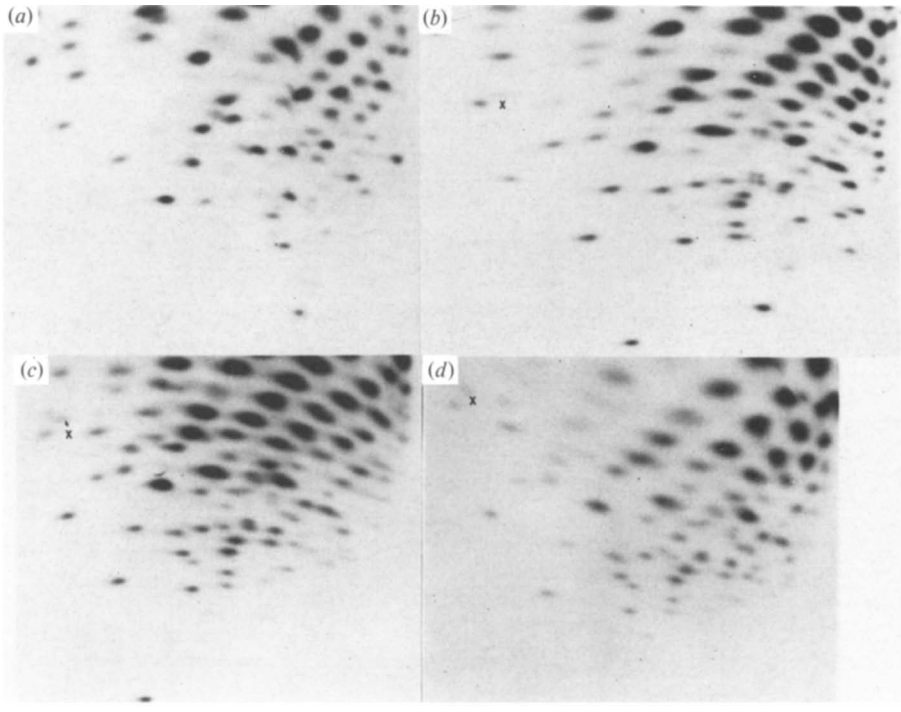


Fig. 6. Oligonucleotide fingerprints of different-sized fractions of poly(A)-containing JHM-DS RNA. (*a*) 20S; (*b*) 35S; (*c*) 45S; (*d*) 55S. The RNA was prepared as described in Fig. 4.

assigned from the 3′ toward the 5′ end. Furthermore, the position of the oligonucleotides on the genome can be estimated from the smallest poly(A)-tagged RNA which contained them (Lai *et al.*, 1981). By this approach, all the oligonucleotides of DL RNA were mapped into several groups on the genome and are presented in Fig. 5. The order of the oligonucleotides within each group is arbitrary. Since the 60S RNA has a mol. wt. of $5{\cdot}4 \times 10^6$ to $6 \times 10^6$ (Lai & Stohlman, 1978; Wege *et al.*, 1978), we assume that this RNA is about 20 kilobases (kb) in length. Therefore, the DL-specific spot, Z, is located about 3 to 5 kb from the 3′ end.

Similar studies were performed with DS RNA. Fig. 6 shows representative fingerprints of different-sized fractions of poly(A)-tagged RNA of DS. From these data it was concluded that a number of the oligonucleotides of DS which are shared with DL were located at approximately the same positions as those of DL. As shown in Fig. 5, the DS-specific spot, X, was mapped at 14 to 15 kb from the 3′ end. Therefore, these two JHM strains differ at two distinct genetic regions.

*Studies with other viruses with different passage history in suckling mouse brain*

To determine the possible genetic origin of the DL and DS variants, we studied the virus pools obtained from the 3rd and 7th suckling mouse brain passages (SMBp3 and SMBp7). SMBp3 is

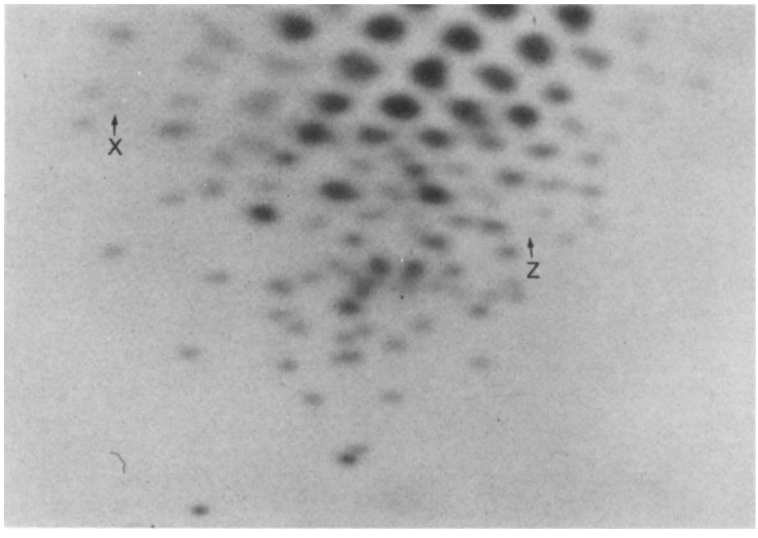


Fig. 7. Oligonucleotide fingerprint of SMBp3. The $^{32}$P-labelled RNA of the uncloned SMBp3 was fingerprinted as in Fig. 2.

Table 2. *Oligonucleotide fingerprints of various JHM variants*

| Virus* | Plaque size | Fingerprints containing X | Fingerprints containing Z | Virulence† (p.f.u./$LD_{50}$) |
|---|---|---|---|---|
| JHM-DL | Large | − | + | 1–5 |
| JHM-DS | Small | + | − | 1000–3000 |
| SMPBp3 | Large–small | + | + | 1–5 |
| SMPBp7 | Intermediate | + | − | 1–5 |
| 3A | Large | + | − | 500–1000 |
| 3B | Large | − | + | 10 |
| 3C | Intermediate | + | − | 100–250 |
| 3D | Large | − | + | 5 |
| 3E | Large | − | + | 1–5 |
| 7A | Intermediate | + | − | 1–5 |
| 7B | Intermediate | + | − | 1–5 |
| 7C | Intermediate | − | + | 1–5 |
| 7D | Intermediate | + | − | 1–5 |

* Viruses 3A to 3E were derived from SMBp3. Viruses 7A to 7D were derived from SMBp7. All of the viruses, except SMBp3 and SMBp7, were cloned.

† Virus at various dilutions was inoculated i.c. into groups of seven mice and the $LD_{50}$ calculated from those animals surviving at 14 days post-infection.

similar to DL in that it causes acute encephalomyelitis with little or no evidence of demyelination while SMBp7 causes encephalomyelitis with primary demyelination in mice (Weiner, 1973; L. P. Weiner, unpublished observation). SMBp7 has homogeneous but intermediate plaque size when assayed on DBT cells (see above). SMBp3, however, produced a wide spectrum of plaque sizes on DBT cells, similar to the range of plaque sizes produced by SMBp8 (Table 2). We studied the genetic composition of these virus pools by oligonucleotide fingerprinting.

The oligonucleotide fingerprints of the uncloned SMBp7 were identical to that of DS, containing the oligonucleotide spot X but not Z (data not shown). In contrast, the oligonucleotide fingerprints of SMBp3 contained approximately equal molar amounts of the oligonucleotide spots X and Z (Fig. 7). Furthermore, these two oligonucleotides are present at about one-half the molar yields of the other oligonucleotides of DL and DS RNAs (Fig. 7). This result suggests that SMBp3 might contain both DL-and DS-like virus particles. To test this

possibility, we studied by oligonucleotide fingerprinting several virus clones obtained from SMBp3 by plaque isolation. Among the five clones we studied, three have oligonucleotide fingerprints similar to that of DL, while two have DS-type fingerprints (Table 2). This result indicates that SMBp3 indeed consists of two virus populations: one similar to DS and the other similar to DL. Surprisingly, one of the clones with a DS fingerprint produced large plaques when assayed on DBT cells. The other clone with a DS-type fingerprint produced intermediate-sized plaques similar to those of SMBp7. We therefore conclude that the oligonucleotide spots, X and Z, are not related to plaque size.

Similar studies have been performed with the virus clones obtained from SMBp7 by plaque isolation. In contrast to those produced by SMBp3, most of the SMBp7 clones studied had DS-type fingerprints (Table 2). However, one of them had a DL-type fingerprint. This exceptional clone yielded intermediate-sized plaques, similar to those produced by the rest of the SMBp7 clones. Our inability to detect the DL-specific oligonucleotide, Z, in the fingerprint of SMBp7 virus pools is most likely due to the low ratio of DL to DS particles. This result confirms that the oligonucleotides X and Z are not related to plaque size.

All of these virus clones are genetically stable. Their plaque sizes remained constant even after repeated passages in tissue culture. We conclude that the plaque size is determined by virus genetic sequences not related to the oligonucleotides X or Z. Since we have various virus isolates with similar oligonucleotide fingerprints but contrasting plaque sizes, we compared DL and DS only with the original isolates from SMBp8. Other virus isolates with similar or different fingerprints or plaque sizes were referred to by the origin of the viruses (Table 2).

To identify the possible functions of these genetic sequences, we have also studied the virulence of all these virus isolates. As shown in Table 2, only viruses 3A and 3C were less virulent than the parental virus pool. The rest of the isolates were as virulent as the DL isolate. Therefore, we concluded that the virulence factor could not be linked to the oligonucleotides X and Z.

## DISCUSSION

In an attempt to understand the molecular basis of virus-induced demyelination, we have begun to search for genetic mutants of the neurotropic JHM strain of murine coronaviruses. In this communication, we reported the isolation and characterization of two plaque-size variants of MHV-JHM. These two variants have very similar *in vitro* biological properties except for plaque size, growth kinetics and virus yield. They also differ in their virulence and pathogenicity: DL is more virulent for mice and produces acute encephalomyelitis with little demyelination, while DS is less virulent and produces primarily a severe acute and chronic demyelination. It is very likely that the small plaque size of DS is a result of its slow growth rate and low virus yield. Therefore, the differences in the *in vitro* biological characteristics of DS and DL are probably determined by the same genetic factor. On the other hand, it is not clear whether the plaque size *in vitro* and the virulence for mice *in vivo* are determined by the same genetic factor. This possibility is suggested by the observations that, in other virus systems, the small-plaque mutants generally are less virulent than large-plaque mutants (Takemoto, 1966). Whether or not the neuropathogenicity of these MHV-JHM variants is determined by a separate genetic factor is also not known.

Oligonucleotide fingerprinting and mapping of the RNA genome suggest that these two variants differ in at least two genetic regions on their genomes: one, represented by the oligonucleotide Z, is located 3 to 5 kb from the 3′ end and the other, represented by the oligonucleotide X, is located 14 to 15 kb from the 3′ end. We previously proposed to divide the genome of mouse hepatitis viruses into seven genetic regions, A to G, based on their mRNA structure (Lai *et al.*, 1981). According to this model, these two genetic regions in the present study will probably represent genes A and E respectively. These two genes presumably code for RNA polymerase and a non-structural protein, p14, respectively (Lai *et al.*, 1981; Siddell *et al.*, 1980). However, the precise localization of these genetic variations will require oligonucleotide fingerprinting studies of mRNA species of these two viruses, experiments which are in progress in our laboratories.

The question arises whether these two oligonucleotide changes are responsible for any of the differences in the biological properties of DL and DS. Since we have been able to isolate, from SMBp3 and SMBp7, JHM variants with DL-type fingerprints which produce small plaques, and JHM viruses with DS-type fingerprints which yield various types of plaques, it can be concluded that these two oligonucleotide changes are not directly correlated with plaque size. By using the same approach we have ruled out the possibility that these sequences are directly or solely responsible for virus virulence. At present, we have not been able to correlate any biological properties with these two genetic regions. Whether they are responsible for the difference in the neuropathogenic property of the virus requires further studies. It is likely that the genetic sequences of these two MHV-JHM variants have additional differences which escape detection by $T_1$ oligonucleotide fingerprinting. These differences might influence any of the biological characters of the virus we have studied.

Although we isolated the original DL and DS variants from SMBp8, it is obvious that these variants were present in earlier virus stocks. Passage of the virus stocks in suckling mouse brain apparently enriched the virus populations with DS genotype. However, the sudden emergence of DL-type viruses in SMBp8 is unaccounted for. Also, although the DL- and DS-type viruses remained genetically stable throughout years of *in vivo* and *in vitro* passages, they appear to have undergone some minor genetic variations which account for the heterogeneity in their plaque size and virulence. This heterogeneity was not reflected in their oligonucleotide fingerprints. It is interesting in this regard to note that the large-plaque and small-plaque variants of alphaviruses behave in a way very similar to our DL and DS variants of JHM virus. In those cases, the plaque morphology and virulence also appear not to be correlated (Strauss & Strauss, 1980).

We wish to thank Chris Patton, and Thomas Hanson for excellent technical help and Josie Lopez and Kipp Mitchell for editorial assistance. This work was supported in part by grant PCM-4567 awarded by the National Science Foundation, grants NS 15079 and CA 16113 awarded by the National Institutes of Health and by a grant 1449-A-1 awarded by the National Multiple Sclerosis Society. J.O.F. is the recipient of postdoctoral fellowship grant FG-544-A-1 from the National Multiple Sclerosis Society.

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(*Received 15 February 1982*)