Genetic Analysis of Murine Hepatitis Virus Strain JHM

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Received 15 September 1981/Accepted 1 February 1982

We performed a genetic analysis of 37 temperature-sensitive mutants of murine hepatitis virus strain JHM. Of our mutants, 32 did not induce murine hepatitis virus-specific RNA synthesis in infected cells at the restrictive temperature, 39°C. By complementation testing we have identified at least seven nonoverlapping complementation groups. Six of the genes identified in this way are required for murine hepatitis virus-specific RNA synthesis. The seventh complementation group is made up of five mutants which induced virus-specific RNA synthesis at 39°C.

The coronaviruses are a group of enveloped, positive-stranded RNA viruses which are pathogenic for their natural hosts (8). One member of the coronavirus group, murine hepatitis virus (MHV), experimentally produces several diseases in mice, depending on the serotype of MHV used, the age and strain of the infected mouse, and the route of inoculation (8). One serotype of MHV, designated MHV-JHM or MHV-4, is highly neurotropic and produces an encephalomyelitis in susceptible mice when it is inoculated intracerebrally or intranasally (1, 10). About 10% of mice survive the acute infection and have widespread demyelinated lesions.

Temperature-sensitive (*ts*) mutants of MHV-JHM have been isolated by several groups of investigators (4, 10; H. Wege, personal communication). In other systems, *ts* mutants have been useful in assigning particular physiological functions to individual viral proteins or viral genes (7). Of special interest, several *ts* mutants of MHV-JHM which differ significantly from the wild-type (WT) virus in the neuropathology that they produce when inoculated into susceptible mice have been described (4, 10). We have been analyzing the mutants isolated by Haspel et al. (4) and Robb et al. (10) by genetic and biochemical means in an attempt to assign specific functions to different viral genes or gene products and particularly to determine whether specific viral genes are responsible for the neurotropism of MHV-JHM and the demyelinated lesions which are observed in infected mice.

In this paper we report the results of complementation tests with our panel of *ts* mutants of MHV-JHM. We have identified seven nonoverlapping complementation groups. Mutants which were defective in their ability to induce

MHV-specific RNA synthesis in infected cells (RNA⁻) can be divided into six complementation groups. Our mutants which induced virus-specific RNA synthesis in infected cells (RNA⁺) complement our RNA⁻ mutants but cannot be further subdivided by complementation testing.

MATERIALS AND METHODS

Cells. The origin of the 17 CL 1 (16) and L2 (12) cell lines has been described previously. Cells were grown at 37°C in 32-ounce (ca. 960-ml) glass prescription bottles in Dulbecco modified Eagle medium (DME) containing 10% newborn calf serum as described previously (2, 9).

Nomenclature of mutants. The nomenclature adopted by Robb et al. (11) for simian virus 40 mutants has been modified here for use with MHV-JHM. The mutants isolated by Haspel et al. (4) will retain their previous numerical designations. Those mutants isolated by Robb et al. (10) after mutagenesis with 5-fluorouracil will be assigned the numbers 101 to 200, with the last two digits of these numbers corresponding to their original numerical designations. For example, the mutant previously identified as 2FU by Robb et al. (10) will now be *ts*102. Those mutants isolated by Robb et al. (10) after mutagenesis with *N*-methyl-*N'*-nitrosoguanidine will be assigned numbers 201 to 300, with the last two digits of these numbers corresponding to their original numerical designations. For example, the N4 mutant will now be *ts*204. We will also indicate the complementation group to which a mutant belongs the first time that it is mentioned in the text (i.e., 5FU will now be referred to as *ts*B105).

Virus. The growth and passage history of the WT MHV-JHM used in these experiments have been described previously (9). The isolation and growth of *ts* mutants of MHV-JHM by Robb et al. (10) and Haspel et al. (4) have been described previously. Mutant stocks were grown at 34°C in 17 CL 1 cells in closed T flasks or glass prescription bottles to avoid cross contamination. It was necessary to reclone the *ts*F203, *ts*A133, and *ts*A137 mutants to obtain working stocks suitable for complementation testing. A total of 30 of the original 34 mutants isolated by Robb et al. (10) and

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7 of 16 mutants isolated by Haspel et al. (4) could be grown to titers of $>1 \times 10^4$ PFU/ml and had efficiencies of plating at 39°C/34°C of $\leq 1 \times 10^{-3}$. These mutants were found to be amenable to genetic analysis.

Mutant stocks with a titer of $<2 \times 10^6$ PFU/ml at 34°C were concentrated by precipitation with polyethylene glycol (17) and suspended by sonic oscillation in DME containing 2% fetal bovine serum to achieve a titer of $>2 \times 10^6$ PFU/ml.

Virus was assayed by plaquing on L2 cells. Confluent monolayers of L2 cells in six-well cluster dishes (3506; Costar, Cambridge, Mass.) were inoculated with 0.2 ml of serial 10-fold dilutions of virus. After virus adsorption for 60 min at room temperature, the monolayer was overlaid with 2.5 ml of DME containing 2% newborn calf serum and 0.8% agarose (Seakem ME; FMC Corp., Rockland, Maine). The cells were incubated for either 2 days at 39°C, the restrictive temperature, or 3 days at 34°C, the permissive temperature. Cells incubated at 34°C were fed on day 2 with 2 ml of DME containing 2% newborn calf serum. Plaques were visualized by gently removing the agarose overlay and staining the monolayer with 0.2% crystal violet in 20% ethanol. All assays were performed at least in duplicate and usually in triplicate.

Complementation testing. To conserve virus stocks, we performed complementation tests in 96-well cluster dishes (3596; Costar). Just-confluent monolayers of 17 CL 1 cells were removed from glass prescription bottles with trypsin, suspended in DME with 10% serum, pelleted by low-speed centrifugation, resuspended in DME containing 2% fetal bovine serum at 5×10^5 cells per ml, and seeded at 5×10^4 cells per well. After 6 h of incubation at 37°C, the medium was removed by aspiration and the cells were inoculated with a 100- μ l mixture of the two mutants being tested or with individual mutants, using a multiplicity of infection (MOI) of 3 for each mutant. Virus adsorption was at 34°C for 1 h. The virus inocula were removed, the monolayers were washed twice with DME, and the medium was replaced with fresh DME containing 2% fetal bovine serum. Incubation in the standard assay was at 39°C until 16 to 18 h postinfection (p.i.). For RNA⁺ mutants and the ts203 mutant it was necessary to shorten the incubation time to 10 to 12 h. Infections were terminated by freezing the cultures at -40°C. The cultures were thawed, and the cells were removed from the plastic by scraping with a plastic micropipette tip. The culture fluids were transferred to tubes containing 0.9 ml of medium, cells were broken by sonic oscillation, and virus titers were determined by plaque assay at 34 and 39°C. Complementation indices (CIs) were calculated using the formula of Fields and Joklik (3): $CI = [(A \times B)_{34^\circ C} - (A \times B)_{39^\circ C}] / [A_{34^\circ C} + B_{34^\circ C}]$. At least two and usually three or more independent experiments were performed for each pair of mutants tested. A CI of >3 was taken to indicate that two mutants belonged to different complementation groups. A CI of between 1 and 3 was felt to be inconclusive, and a CI of <1 was taken to mean that two mutants belonged to the same complementation group. In all experiments a well was infected with WT virus at the same MOI, and the yield was determined to calculate the percentage of WT yield for any pair of complementing mutants. The yield of WT virus was 2×10^5 to 2×10^6 PFU per culture.

Labeling, extraction, and gel electrophoresis of intracellular RNA. 17 CL 1 cells were infected at an MOI of 0.1 as described previously (5) and incubated at 39 or 34°C in phosphate-free DME. Cells were labeled with 125 μ Ci of 32 P_i in the presence of 5 μ g of actinomycin D per ml from 9 to 12 h p.i. at 34°C and 6 to 8 h p.i. at 39°C. Cytoplasmic extracts were prepared by lysing the cells in 0.01 M Tris-0.01 M NaCl-0.0015 M MgCl₂-0.5% Nonidet P-40-0.1% sodium dodecyl sulfate. These extracts were adjusted to 1% sodium dodecyl sulfate-0.4 M NaCl-0.01 M EDTA, digested with 1 mg of proteinase K per ml, extracted with phenol-chloroform, and precipitated with ethanol as described previously (5). The nucleic acids were collected by centrifugation, denatured with glyoxal, and analyzed by agarose gel electrophoresis (5, 6).

RESULTS

Complementation testing. Prior work had shown that cultures infected with a mixture of tsA204 and tsB105 and incubated at 39°C developed cytopathic effect and contained viral antigens, whereas cultures singly infected with either of these mutants did not (J. Robb, personal communication). With this as a starting point, preliminary experiments in 17 CL 1, L2, and DBT cells at MOIs of 1, 3, and 5 indicated that optimal complementation occurred in 17 CL 1 cells at an MOI of 3 (data not shown). The efficiency of complementation between the tsA204 and tsB105 mutants was host cell dependent. 17 CL 1 cells simultaneously infected with these two mutants and incubated at 39°C yielded two- to fivefold-more virus than did similarly infected cultures of L2 or DBT cells. This was probably because in our hands WT and ts mutants of MHV-JHM grow to a two- to fivefold-greater titer in 17 CL 1 cells than in DBT or L2 cells. We therefore adopted an MOI of 3 in 17 CL 1 cells as our standard conditions.

We then proceeded to group our mutants by performing a series of pairwise complementation tests. It quickly became apparent that for cultures mixedly infected with any two RNA⁻ mutants the appearance of syncytia was indicative of complementation (Fig. 1). Cultures infected with complementing pairs of mutants, tsA201 and tsE248 or tsA204 and tsE248, developed typical MHV cytopathology, whereas cultures infected with tsA204 and tsA201 or singly infected with these mutants developed no cytopathology. Complementation analysis of 32 RNA⁻ mutants unambiguously identified six nonoverlapping complementation groups. The data from complementation tests with 13 mutants are shown in Table 1. CIs of as high as 1,400 were obtained with some pairs of mutants. Yields of virus in crosses between complementing RNA⁻ mutants ranged from 0.2 to 32% of the yield of WT virus. In no case was the yield of virus greater than 0.06% of WT when two mu-

TABLE 1. CIs of 10 RNA⁻ mutants

Group	Mutant	CI ^a												
		<i>ts</i> 204	<i>ts</i> 116	<i>ts</i> 124	<i>ts</i> 105	<i>ts</i> 216	<i>ts</i> 260	<i>ts</i> 106	<i>ts</i> 264	<i>ts</i> 129	<i>ts</i> 103	<i>ts</i> 131	<i>ts</i> 248	<i>ts</i> 203
A	<i>ts</i> 204		<1 (0.001)	<1 (0.001)	1,200 (11)	15 (0.8)	370 (2.9)	720 (4.9)	150 (2.9)	990 (22)	120 (2.1)	370 (16)	140 (29)	160 (8.6)
	<i>ts</i> 116			<1 (0.001)	250 (0.9)	59 (0.8)	38 (0.2)	110 (0.8)	320 (1.9)	240 (5.3)	71 (0.9)	1,100 (8.7)	180 (5.8)	14 (7.4)
	<i>ts</i> 124				610 (2.3)	53 (0.7)	310 (1.1)	230 (3.4)	76 (0.3)	7 (0.2)	65 (0.9)	940 (9.2)	130 (2.6)	18 (8.5)
B	<i>ts</i> 105					<1 (0.003)	750 (6.2)	1,200 (19)	1,200 (10)	780 (19)	240 (0.9)	560 (17)	1,400 (13)	370 (11)
	<i>ts</i> 216						110 (1.5)	110 (1.1)	17 (0.6)	390 (7.3)	130 (1.8)	170 (10)	34 (8.5)	46 (21)
C	<i>ts</i> 260							<1 (0.001)	250 (1.8)	1,300 (26)	710 (9.2)	330 (20)	91 (17)	50 (5.8)
	<i>ts</i> 106								550 (7.0)	650 (12)	1,400 (11)	90 (6.0)	210 (7.1)	320 (13)
D	<i>ts</i> 264									<1 (0.001)	<1 (0.003)	<1 (0.002)	96 (20)	69 (6.1)
	<i>ts</i> 129										<1 (0.03)	<1 (0.01)	190 (32)	14 (7.0)
	<i>ts</i> 103											<1 (0.06)	100 (20)	24 (11)
E	<i>ts</i> 131												50 (14)	14 (11)
	<i>ts</i> 248													370 (8.9)
F	<i>ts</i> 203													

^a Mean values of at least two experiments. Numbers within parentheses are percentages of the yield obtained from a parallel infection with the WT virus.

FIG. 1. Cytopathology produced by complementing mutants. Cells were infected and incubated at 39°C as in the standard complementation test described in the text. Cultures were mixedly infected with *ts*202 and *ts*248 (A), *ts*204 and *ts*248 (B), and *ts*202 and *ts*204 (C). Cultures were singly infected with *ts*202 (D), *ts*204 (E), and *ts*248 (F). Photomicrographs were taken at 15 h p.i. at an original magnification of $\times 100$.

tants belonged to the same complementation group. Greater than 95% of the yield of cultures infected with two complementing mutants was temperature sensitive as shown by the efficiency of plating and by testing the progeny of single plaques picked at 34°C for temperature sensitivity. Experiments designed to detect recombination in cultures mixedly infected at 34°C with pairs of mutants have failed to do so. A portion (1 to 5%) of the yield from cultures infected with some pairs of complementing mutants produced plaques at 39°C. However, the progeny from these plaques was still temperature sensitive. These plaques could have arisen from polyploid virus particles producing complementation during the plaque assay. Most of our mutants (59%) belonged to a single complementation group, designated group A (Table 2).

In contrast to the high efficiency of complementation among RNA⁻ mutants, complementation tests between our five RNA⁺ mutants and six prototype RNA⁻ mutants resulted in CIs of

between 3.1 and 100, with virus yields of 0.2 to 9.4% of that obtained in parallel infections with WT virus (Table 3). However, it was clear that all five RNA⁺ mutants belonged to a complementation group(s) different from our RNA⁻ mutants. Experiments to identify more than one complementation group within our collection of five RNA⁺ mutants were inconclusive (Table 4). No complementation was observed between *ts*G104, *ts*G110, *ts*G115, and *ts*G8. One RNA⁺ mutant, *ts*G15, gave equivocal CIs with *ts*G104 and *ts*G8.

The variability in our complementation test was often quite high for pairs of mutants which complemented efficiently. As an extreme example, three independent experiments with *ts*C260 and *ts*B104 had CIs of 240, 1,200, and 800, with a mean CI of 750 and a standard deviation of 480 (Table 1). This variability was less for mutants which complemented with a lower efficiency. Two independent complementation tests with *ts*B216 and *ts*D264, for example, resulted in CIs

TABLE 2. Mutants used in this study

Group	RNA phenotype	Mutant(s)
A	-	<i>ts102, ts109, ts116, ts117, ts120, ts124, ts133, ts137, ts138, ts201, ts202, ts204, ts207, ts218, ts232, ts270, ts271, ts7, ts10, ts11, ts12, ts16</i>
B	-	<i>ts105, ts216</i>
C	-	<i>ts106, ts260</i>
D	-	<i>ts103, ts129, ts131, ts264</i>
E	-	<i>ts248</i>
F	-	<i>ts203</i>
G	+	<i>ts104, ts110, ts115, ts8, ts15</i>

of 22 and 12, with a standard deviation of 7. The variability in this assay for mutants which did not complement was small. The variability in the assay appeared to be associated with the variability of the efficiency of complementation, i.e., the yield of virus obtained in the mixedly infected cultures which could vary as much as 10-fold from experiment to experiment, as well as a smaller variability in the leak yields from the single-infection controls. It was not due to a background of uneclipsed virus, which was below 50 PFU per culture for WT virus and for the *ts* mutants that we examined. The assignment of mutants to a given complementation group was not affected by the variability in our complementation assay, with one possible exception. The *tsG15* mutant had a mean CI of 1.9 with *tsG8* calculated from four independent experiments. However, the standard deviation of the data from these experiments was 1.0. In this case it is possible, although we feel that it is unlikely, that the variation in the assay obscured a low level of complementation.

Gel analysis of virus-specific RNA. MHV-infected cells have recently been shown to contain seven virus-specific RNA species (5, 14). With this fact in mind, we analyzed, by agarose gel

TABLE 4. Complementation test of RNA⁺ mutants

Mutant	CI ^a				
	<i>ts104</i>	<i>ts110</i>	<i>ts115</i>	<i>ts8</i>	<i>ts15</i>
<i>ts104</i>		0.3 (0.1)	0.0 (0.3)	0.5 (0.2)	1.8 (0.2)
<i>ts110</i>			0.0 (0.6)	0.8 (0.8)	0.8 (0.4)
<i>ts115</i>				0.4 (0.8)	0.0 (0.5)
<i>ts8</i>					1.9 (0.4)
<i>ts15</i>					

^a Mean values of at least two experiments. Numbers within parentheses are percentages of the yield obtained from a parallel infection with the WT virus.

electrophoresis, cultures infected with the five mutants in our collection which had been previously classified as being RNA⁺ on the basis of their ability to induce infected cultures to incorporate [³H]uridine at 39°C (10; M. V. Haspel, unpublished data). Duplicate sets of cultures were infected with WT virus or with one of our five RNA⁺ mutants. One set of cultures was incubated at 39°C and the other set was incubated at 34°C. Cultures were labeled with ³²P_i in the presence of actinomycin D from 6 to 8 h p.i. at 39°C and from 9 to 12 h p.i. at 34°C, and the cytoplasmic RNA was extracted and analyzed by agarose gel electrophoresis (Fig. 2). Cells infected under restrictive (39°C) or permissive (34°C) conditions with either WT virus or any of our RNA⁺ mutants synthesized the seven major MHV-specific RNAs (RNAs 1 to 7) and one additional MHV-specific RNA (asterisk, Fig. 2), which we have noted occasionally in the past (5).

We also similarly examined representative mutants from complementation groups A to F which had previously been categorized as being RNA⁻ or RNA intermediate (10; Haspel, unpublished data). Of the 14 mutants which we had previously classified as having an RNA-intermediate phenotype (10), 3 either were too leaky or could not be grown to a sufficiently high titer and therefore were not examined further. We performed a gel analysis with three RNA-intermediate mutants (*ts204*, *ts218*, and *ts131*) which induced relatively large amounts of MHV-specific RNA at 39°C when assayed by actinomycin D-

TABLE 3. Complementation of RNA⁻ with RNA⁺ mutants (groups A to F)

RNA ⁺	CI ^a with RNA ⁻ :					
	<i>ts204</i>	<i>ts105</i>	<i>ts106</i>	<i>ts264</i>	<i>ts248</i>	<i>ts203</i>
<i>ts104</i>	7.3 (0.3)	5.0 (0.2)	5.7 (0.2)	7.0 (0.3)	5.3 (0.2)	30 (1.3)
<i>ts110</i>	36 (0.8)	23 (0.7)	9.5 (0.3)	6.5 (0.3)	64 (2.2)	29 (6.0)
<i>ts115</i>	11 (4.9)	5.3 (1.6)	3.1 (1.0)	5.1 (1.2)	6.3 (1.9)	60 (5.0)
<i>ts8</i>	25 (2.3)	24 (2.3)	22 (3.6)	19 (1.9)	31 (4.0)	100 (4.7)
<i>ts15</i>	15 (3.5)	29 (3.8)	57 (9.4)	62 (4.6)	39 (3.2)	110 (5.7)

^a Mean values of at least two experiments. Numbers within parentheses are percentages of the yield obtained from a parallel infection with the WT virus.

FIG. 2. Analysis of MHV-specific RNA from cultures infected with RNA⁺ mutants. Cultures were infected with WT virus or *ts* mutants, incubated at 34 or 39°C, and labeled with ³²P_i, and the cytoplasmic RNA was extracted and analyzed by electrophoresis in 0.8% agarose gels as described in the text. The virus used and the incubation temperature of each culture are indicated in the figure. The figure is a composite of two different exposures of the same gel.

resistant [³H]uridine incorporation. Neither *ts*204 (Fig. 3) nor *ts*218 (Fig. 4) induced the synthesis of detectable MHV-specific RNA at 39°C, although parallel cultures at 34°C did synthesize MHV-specific RNA. A similar result was obtained for *ts*131 (data not shown). We also examined by gel analysis 9 of the 22 mutants previously characterized as RNA⁻ on the basis of actinomycin D-resistant [³H]uridine incorporation. None of these mutants induced detectable MHV-specific RNA synthesis at 39°C. The data for six of these mutants (*ts*248, *ts*264, *ts*106, *ts*105, *ts*260, and *ts*203) are shown in Fig. 3 and 4. It thus appears that our previous designation of several of these mutants (*ts*204, *ts*218) as RNA intermediate, based upon actinomycin D-resistant [³H]uridine incorporation, is not justified. The heterogeneous smear observed in some cultures labeled at 39°C (Fig. 3) represents DNA which is occasionally carried through our extraction procedures.

DISCUSSION

The experiments described above have allowed us to identify six complementation groups containing mutants which are defective in their ability to induce virus-specific RNA synthesis and one additional complementation group made up of mutants in a gene or genes not required for

the synthesis of MHV-specific RNA. The vast majority of our mutants (32 of 37) contain mutations in genes required for RNA synthesis. Of these 32 RNA⁻ mutants, 22 belong to a single complementation group, whereas the 10 remaining RNA⁻ mutants comprise five additional complementation groups. These results parallel in some respects those obtained with another positive-stranded virus, Sindbis virus (15). Sindbis virus, an alphavirus, has been shown by complementation testing to contain four genes which are required for virus-specific RNA synthesis. In addition, most of the RNA⁻ mutants of Sindbis virus also belong to a single complementation group. The genome of MHV is 6.1×10^6 daltons in size (5). The 3' 3.4×10^6 daltons of the genome is expressed late in infection (4 h p.i.) and is probably not required for RNA synthesis (5). The remaining 5' 2.7×10^6 daltons of the genome is sufficiently large to encompass six gene functions required for virus-specific RNA synthesis which have been tentatively identified by complementation testing. Although it is possible that some of the six RNA⁻ complementation groups that we have identified are due to intracistronic complementation, we feel that this is unlikely. Intracistronic complementation is usually weak, unlike the efficient complementation between RNA⁻ mutants which we observed. A firm determination of the number of

FIG. 3. Analysis of MHV-specific RNA from cultures infected with RNA⁻ mutants belonging to complementation groups A to E. Cells were infected with *ts248* (group E), *ts264* (group D), *ts106* (group C), *ts105* (group B), or *ts204* (group A), incubated at 34 or 39°C and labeled with ³²P_i, and the cytoplasmic RNA was extracted and analyzed as described in the text.

genes required for MHV-specific RNA synthesis requires further biochemical data and the development of in vitro systems for the synthesis of MHV-specific RNA.

The five RNA⁺ mutants in our collection complemented all six RNA⁻ complementation groups, although at a relatively low efficiency. This low efficiency of complementation is some-

FIG. 4. Analysis of MHV-specific RNA from cultures infected with *ts203*, *ts260*, and *ts218*. Cells were infected with WT virus, *ts203* (group F), *ts260* (group C), or *ts218* (group A), incubated at 34 or 39°C, and labeled with ³²P_i, and the cytoplasmic RNA was extracted and analyzed as described in the text. The figure is a composite of two different gel analyses.

what surprising since RNA⁺ mutants of Sindbis virus efficiently complement RNA⁻ mutants (15). We could not further subdivide our RNA⁺ mutants into different complementation groups, although the data suggest that *ts15* might contain a mutation in a different gene from the other four RNA⁺ mutants. The failure of any of our five RNA⁺ mutants to complement each other does not imply that all of these mutations are necessarily in the same gene. However, the three RNA⁺ mutants (*ts104*, *ts110*, and *ts115*) isolated by Robb et al. (10) were originally selected on the basis of their inability to induce syncytium formation at 39°C and could conceivably be mutations in a single gene responsible for MHV-induced cell fusion. Although the other two RNA⁺ mutants (*ts8* and *ts15*) were selected on the basis of their ability to form plaques at 34°C but not at 39°C (4) and are theoretically less likely to be restricted to a single gene, these mutants also fail to induce syncytium formation at 39°C and could contain defects in the same gene as the mutants of Robb et al. Biochemical evidence suggests that there are at least six MHV genes expressed late (after 4 h p.i.) in infection (2, 5, 13, 14) which are probably not required for RNA synthesis. A much larger number of RNA⁺ mutants need to be isolated to obtain mutants in all of these genes. By studying a larger number of RNA⁺ mutants by genetic, biochemical, and immunological means, it should be possible to identify mutations in all of the MHV late genes and to determine their physiological function in infected cells.

Finally, it should be noted that each complementation group is not necessarily homogeneous in regard to the neuropathology produced by the members of that group. Complementation group A contains mutants which are completely attenuated and produce no disease (for example *ts204*), mutants which produce an acute encephalomyelitis similar to that produced by WT MHV-JHM (for example, *ts232*), and a mutant which produces a chronic meningoencephalitis with demyelination (*ts202*) (10). Conversely, mutants in different complementation groups can produce a similar pathology. It is, therefore, probably incorrect at this time to ascribe a specific pathological lesion to a specific MHV-JHM gene during infection with this virus. However, this does not detract from the usefulness of several of these mutants in studying virus-induced demyelination.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the National Multiple Sclerosis Society and by Public Health Service grants NS 15211, NS 14068, and NS 12428 from the National Institute of Neurological and Communicative Disorders and Stroke. J.L.L. is the recipient of Teacher Investiga-

tor Award NS 00418 from the National Institute of Neurological and Communicative Disorders and Strokes.

We thank Eric Paavola for excellent technical assistance and Frances Doolittle and Pat Ford for aid in preparing the manuscript.

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