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## Characterization of the Cold-Sensitive Murine Hepatitis Virus Mutants Rescued from Latently Infected Cells by Cell Fusion

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Mouse hepatitis virus, a coronavirus, was rescued from latently infected mouse neuroblastoma cells by polyethylene glycol-induced fusion to permissive cells. The isolated viruses grew to equal or higher titers than parental JHM virus at 39° but were restricted in replication at 32° and hence were cold-sensitive mutants. Neither isolate synthesized RNA at the nonpermissive temperature (32°), and temperature shift experiments indicated that the restrictive cold-sensitive step in replication occurred during an early viral function. The isolates were unable to complement each other at 32°. Although coinfection between each mutant and parental JHM virus often resulted in decreased yields of both infecting viruses, interference could not be convincingly demonstrated. Both mutants were more thermostable than parental JHM virus and could be distinguished from each other and from parental JHM virus by their thermal inactivation kinetics. The isolates were obtained from single cell clones and therefore presumed to be homogeneous; however, thermal inactivation of one (S-3JHM) indicated the possible existence of a second population. Subclones of virus prepared from each isolate were also found to be thermally stable and to retain the cold sensitive defect. No indication of a second population of S-3JHM virus was detected.

### INTRODUCTION

The understanding of persistent viral infections of the central nervous system has become increasingly important in recent years. Persistent viral infections have been implicated in such human diseases of the central nervous system as subacute sclerosing panencephalitis, progressive multifocal leukoencephalopathy, and multiple sclerosis (Johnson and Herndon, 1974; ter Meulen *et al.*, 1970; Weiner and Narayan, 1974). Infection of mice with the neurotropic JHM strain of murine hepatitis virus (MHV), a coronavirus, represents an animal model of a persistent, viral-induced demyelinating disease of the central nervous system. Although demyelination occurs during the

acute infection (Weiner, 1973), of greater interest is the mechanism by which JHM virus establishes a latent infection whose hallmark is chronic and recurrent myelin loss (Herndon *et al.*, 1975).

In analyzing the interactions of JHM virus with cells derived from the mouse nervous system, we have shown previously that JHM virus can establish a persistent infection in mouse neuroblastoma cells (Stohlman and Weiner, 1978). These chronically infected cells were studied to detect possible changes in the virus population which could be related to the ability of JHM virus to persist *in vitro* and *in vivo*. By several criteria, the infectious virus released by the chronically infected cells was indistinguishable from parental virus. Maintenance of the infection did not appear to be due to the selection of temperature-sensitive (*ts*) mutants or to the production of defective interfering particles or interferon. Infectious virus, viral cell surface antigen, and cytopathic effect (CPE) were lost from chronically infected cells after passage in the

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presence of antiviral antibody although intracellular viral antigen persisted. No release of infectious virus was detected after removal of the antiviral antibody. After cloning chronically infected neuroblastoma cells in the presence of antiviral antibody, some clones retained viral antigen in the cellular cytoplasm while others did not. No infectious virus could be induced in any of the cell clones by a variety of chemical or physical treatments or by cocultivation with cells susceptible to JHM virus (Weiner and Stohlman, 1978). Virus was recovered following the fusion of clones derived from single cells to permissive cells by polyethylene glycol (Weiner and Stohlman, 1978). We describe here the characterization of the viruses isolated from latently infected mouse neuroblastoma cells.

#### MATERIALS AND METHODS

*Virus isolation.* The N<sub>1</sub> cell line is a mouse neuroblastoma cell line chronically infected with JHM virus (Stohlman and Weiner, 1978). Single cell clones were obtained in the presence of 1% heat-inactivated (56° for 30 min) anti-JHM virus hyperimmune ascitic fluid (plaque reduction neutralization titer = 1/5000).

Virus was isolated from these non-virus-producing cell clones by fusion with polyethylene glycol (PEG) to either DBT (Hirano *et al.*, 1974) or 17 clone 1 (17CL1) (Sturman and Takamoto, 1972) cell lines by the method of Pontecorvo (1975). Briefly, cocultivated cultures were washed twice with Dulbecco's minimum essential medium (DMEM) without serum, 1.0 ml of a sterile 50% solution of PEG (mw 6000, Koch Light) in DMEM, pH 7.8, was added to each plate with agitation for 1 min at room temperature. Excess PEG was removed, and 2.0 ml of DMEM was added. At 1-min intervals, 0.2-, 0.2-, 0.2-, 0.5-, 1.0-, and 2.0-ml volumes of DMEM were added and mixed thoroughly. The DMEM was removed 1 min after the final addition and replaced with 5.0 ml of DMEM containing 10% fetal calf serum (FCS). After 18 hr incubation, the medium was removed and replaced with 5.0 ml of DMEM containing 2% FCS. The plates were further incubated for 96 hr and checked for

CPE. Supernatants containing virus were removed from cultures showing viral CPE at 96 hr postfusion.

*Preparation of virus subclones.* The original fluid from the fused cultures was used as starting material for the preparation of subclones. Clones were plaque purified three times in DBT cells. Isolated plaques were recovered and propagated in DBT cells. Following the third plaque purification, pools were prepared by infecting DBT cells at an m.o.i. of 0.001. These pools were used for all studies described.

*Thermal inactivation.* Thermal inactivation studies were performed at 42°. Parent stocks of the virus isolated from the original fusion plates and plaque-purified clones were tested. Virus was adjusted to approximately 10<sup>5</sup> PFU/ml in phosphate buffered saline, pH 7.2 (PBS) containing 10% heat-inactivated,  $\gamma$ -globulin-free FCS. At various times from 15 to 180 min, samples were removed, flash frozen in dry ice-acetone, and stored at -70°. Residual infectivity was determined by plaque assay on DBT cells at 37° as previously described (Stohlman and Weiner, 1978).

*Complementation.* Complementation experiments were carried out by infecting DBT cells at a multiplicity of infection of 10 PFU per cell. Following adsorption for 60 min at 32°, the cells were washed twice with medium and incubated for 14 hr at 32°. Supernatants were assayed for infectious virus at 37°. For experiments in which JHM virus was tested, the DL clone of JHM virus (Lai and Stohlman, 1978) was used at an m.o.i. of 1.0. The complementation index was calculated as the ratio: yield from the mixed infection at the restrictive temperature (32°) divided by the sum of the yields from the single infections at 32°. The yields were assayed at 37°; results were identical to assays at 39° except that the plaques were better defined.

*Virus-specific RNA synthesis.* DBT cells were used to determine virus-specified RNA synthesis. Preliminary experiments showed that infection at an m.o.i. of 1.0 resulted in approximately 80% of the cells scoring as infectious centers (Stohlman and Weiner, 1978). Monolayers of DBT cells in 16-mm-diameter wells were infected at an m.o.i. of

approximately 10 for 1 hr at room temperature. After removal of the inoculum, 1 ml of warm DMEM containing 2% FCS was added, and duplicate cultures were incubated at either 32 or 39° for 1 hr. The medium was removed, the cultures were washed one time with medium, and finally 1 ml of DMEM containing 2% FCS, 2  $\mu\text{g/ml}$  actinomycin D (a gift of Merck, Sharpe, and Dohme), and 5  $\mu\text{Ci}$  of [5- $^3\text{H}$ ]uridine/ml was added. Following incubation for an additional 14 hr, the medium was removed, then cells were washed twice with cold PBS, and the monolayers were solubilized with 2% (w/v) sodium lauryl sulfate in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS. An equal volume of 20% trichloroacetic acid was added and the precipitate collected by filtration on Whatman GF/C glass-fiber filters after at least 8 hr at 4°. The filters were dried and counted as previously described (Lai and Stohlgman, 1978). Clones were assigned an RNA<sup>-</sup> phenotype if they synthesized less than 20% of the virus specific RNA at 32° compared to the amount synthesized at 39°.

*Temperature shift experiments.* DBT cell monolayers were infected at an m.o.i. of approximately 5. Following incubation for 60 min at room temperature, unabsorbed virus was removed by two rinses with serum-free DMEM, and the plates were fed with 2.0 ml of prewarmed DMEM containing 2% FCS. Cultures were immediately placed at either 32 or 39° and shifted to the alternate temperature at 1, 2, 4, 6, 8, or 10 hr after the start of the experiment. At each temperature shift time point, samples of supernatants were taken for virus assay. The plates were then further incubated at the new temperature for a total of 12 hr from the start of the experiment. The plates were again sampled at the termination of the experiment. Virus samples were titered on monolayers of DBT cells at 37° as previously described (Stohlgman and Weiner, 1978).

## RESULTS

### *Rescue of Infectious Virus*

Single cell clones isolated from the N<sub>1</sub> cell line did not exhibit viral CPE during subculture and were morphologically indistinguishable from clones isolated from

uninfected mouse neuroblastoma cells (Stohlgman and Weiner, 1978; Weiner and Stohlgman, 1978). Infectious virus was not detected in supernatant fluids from the cell clones following incubation at 32, 37, or 39° for 72 hr or after cocultivation with either DBT or 17CL1 cells for 72 hr at these three temperatures.

Virus was recovered by PEG-mediated fusion of S-1 and S-3 cell clones with DBT or 17CL1 cells (Pontecorvo, 1975; Weiner and Stohlgman, 1978). Clone S-1 contained JHM viral antigen in the cytoplasm detectable by immunofluorescence while clone S-3 did not. Infectious virus was rescued from S-1 cells in five of seven attempts and from S-3 cells in three of six attempts using either DBT or 17CL1 cells (Stohlgman *et al.*, 1979). Homopolykaryons or heteropolykaryons of DBT and 17CL1 cells did not yield infectious virus indicating that the rescued virus populations were derived from the S-1 and S-3 cell clones. These viruses have been designated S-1JHM virus and S-3JHM virus (Weiner and Stohlgman, 1978).

### *Properties of the Rescued Virus*

The viruses recovered from both cell clones by PEG fusion were serologically indistinguishable from JHM virus and replicated, producing typical CPE, in cell lines that support JHM virus growth. Plaque size of both S-1JHM virus and S-3JHM virus was identical in DBT and 17CL1 cells. In contrast, both parental JHM virus and the virus released by the chronically infected neuroblastoma cells produce heterogeneous plaque sizes in DBT and 17CL1 cells (Stohlgman and Weiner, 1978). Plaque size of S-1JHM virus and S-3JHM virus remained stable through several passages in DBT cells, and S-1JHM virus plaque size was also not altered by *in vivo* brain passage in C57BL mice.

Since persistent infections often result in the appearance of *ts* mutants (Rima and Martin, 1976), the rescued viruses were tested for their growth at the extremes of the temperature range that are nonrestrictive to the growth of parental JHM virus (Stohlgman and Weiner, 1978), i.e., 32 and 39°. Both

TABLE 1

GROWTH OF JHM VIRUS, S-1JHM, AND S-3JHM VIRUSES ISOLATED FROM LATENTLY INFECTED CELL CLONES AT 32, 37, AND 39°

Temperature (°)	Titer (PFU/ml) <sup>a</sup>		
	JHM	S-1JHM	S-3JHM
32	$1.0 \times 10^5$	$1.0 \times 10^3$	$2.0 \times 10^2$
37	$2.0 \times 10^5$	$8.0 \times 10^5$	$2.0 \times 10^5$
39	$1.0 \times 10^5$	$1.0 \times 10^6$	$2.0 \times 10^6$

<sup>a</sup> Supernatant virus harvested at 18 hr postinfection.

S-1JHM virus and S-3JHM virus were restricted in replication at 32° while the yield of infectious virus at 37° was comparable to that of JHM virus (Table 1). These viruses also grew better than parental JHM virus at 39° and are therefore classified as cold-sensitive (*cs*) mutants. Although the upper temperature limit for efficient replication of JHM virus is 39°, the two *cs* mutants were examined for their ability to replicate efficiently at even higher temperatures. Neither S-1JHM nor S-3JHM viruses replicated as efficiently at 40° as they had at 39°. Both were inhibited to about the same extent, i.e., 90% at 40° and 99% at 41°, as parental JHM virus.

Since the rescued viruses could be distinguished by neither plaque morphology nor growth at 32°, thermal inactivation studies were performed. As can be seen from Fig. 1A, JHM virus is rapidly inactivated at 42°, displaying linear inactivation kinetics. More than 90% of the infectivity was lost within 30 min. S-1JHM virus, however, is considerably more stable at 42° than JHM virus, requiring more than 120 min before 90% of the infectious virus is inactivated.

Inactivation of S-3JHM virus yielded a two-component inactivation curve (Fig. 1B), suggesting that it might be composed of more than a single population. The slope of the curve for the less heat-stable component was suggestive of that of parental JHM virus while the slope of the more heat-stable component was similar to the curve of S-1JHM virus (Fig. 1A). To determine if S-3JHM virus was composed of two virus populations with different rates of thermal

inactivation, it was heated at 42° for 2 hr, and then the surviving virus was propagated in DBT cells at 37°. The heat-treated S-3JHM virus displayed linear inactivation kinetics, but the slope of the curve resembled neither of the slopes of the original inactivation curve and was intermediate between the two (Fig. 1B).

S-1JHM and S-3JHM viruses were respectively derived from cells originating from a single cell and therefore should be very similar if not identical. However, the two-component thermal inactivation of S-3JHM virus indicated the possible existence of two populations in this pool. Thrice derived subclones were prepared from both S-1JHM and S-3JHM viruses. These subclones are designated so that the first digit indicated the parent of derivation, i.e., *cs11* is the number one subclone from S-1JHM virus while *cs31* is the number one subclone of S-3JHM virus. All of the subclones retained the *cs* marker and were more thermally stable than JHM virus. JHM virus was inactivated at a rate of 5.32  $\log_{10}/\text{hr}$ . The geometric mean inactivation rate of the clones derived from S-1JHM virus was 2.22  $\log_{10}/\text{hr}$  while the rate for the S-3JHM virus clones was 1.57  $\log_{10}/\text{hr}$ . None of the subclones of S-3JHM virus were as thermolabile as JHM virus. No evidence that the original S-3JHM virus pool contained a subpopulation yielding altered plaque size or a population of non-*cs* virions was found by testing an additional 156 virus clones for their ability to form plaques at 32 and 39°.

#### RNA Synthesis

The ability of the subclones derived from S-1 and S-3 JHM viruses to synthesize virus-specific RNA at the nonpermissive temperature (32°) was tested. All 15 subclones, 7 derived from S-1 JHM virus and 8 from S-3 JHM virus, had retained the *cs* marker, and none were able to synthesize RNA at the restrictive temperature. The average percentage RNA synthesized at 32° by all the subclones was 10.74% of that synthesized at 39°. Any value less than 20% was considered to be consistent with an

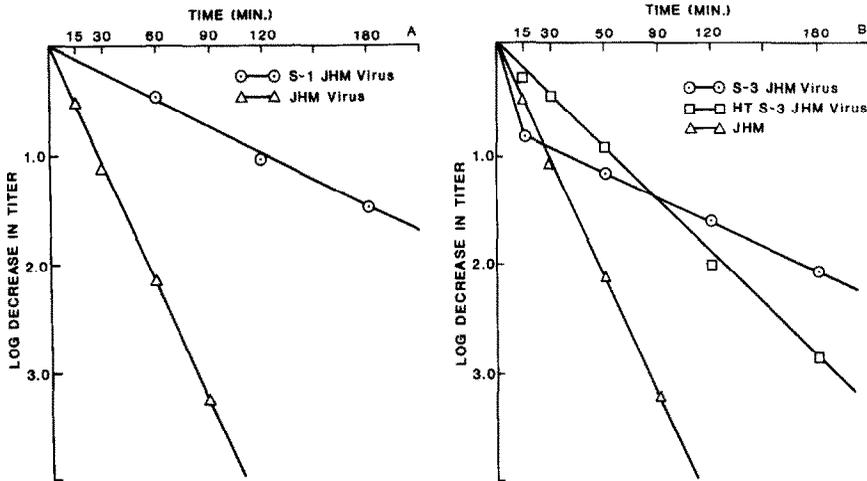


FIG. 1. Thermal inactivation of the infectivity of JHM virus, S-1JHM virus, and S-3JHM virus at 42°. (A) Comparison of JHM virus with S-1JHM virus. (B) Comparison of JHM virus with S-3JHM virus and the S-3JHM virus thermally stable component.

RNA<sup>-</sup> phenotype. The highest value obtained was 18.0% and the lowest was 1.6%. Parental JHM virus synthesized 90% of the RNA at 32° that was synthesized at 39°.

#### Temperature Shift Experiments

Temperature shift experiments were carried out as described to determine if the *cs* defect at 32° was an early or late function in virus replication. Figure 2 shows the results of experiments with S-1JHM virus subclone *cs*11 and parental JHM virus when the temperature shift was from the permissive (39°) to the nonpermissive (32°) temperature. Supernatant virus was removed from culture at the time of each shift and again from the same culture at 12 hr postinfection when the experiment was terminated. A normal yield of JHM virus was obtained after all temperature shifts (data not shown). Although not completely restricted at the nonpermissive temperature (32°C), *cs*11 replication was suppressed when cultures were shifted to 32° before 6 hr. The amount of virus found at 12 hr in cultures shifted at 6 hr probably reflects synthesis of new virus at the permissive temperature since it is evident from the data in Fig. 3 that little virus is released in cultures held 6 hr at 32°. When shifted from the nonpermissive (32°) to the permissive

(39°) temperature, the replication of *cs*11 virus was not inhibited in cultures shifted at 1, 2, or 4 hr postinfection (Fig. 3). However, replication was suppressed in cultures transferred after 4 hr. The titers obtained from the cultures shifted at 8 and 10 hr postinfection indicate that by 8 hr some virus replication could take place. By 10 hr postinfection, the yield of virus was less than the yield obtained from cultures shifted at 8 hr postinfection, indicating that the block in replication must be early since no new virus could be synthesized during this period. We

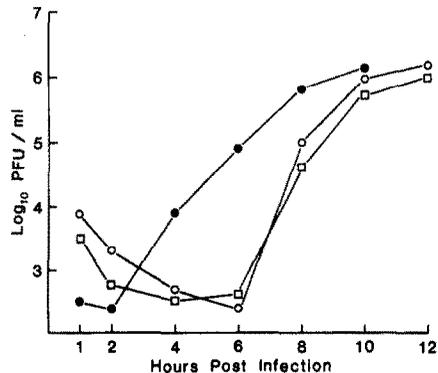


FIG. 2. The yields of virus from cultures infected with S-1JHM virus (○) after incubation at 39° and the yield of S-1JHM virus at 12 hr postinfection following shift to the nonpermissive temperature (32°) at the times indicated (●). Growth of JHM virus at 39° (□).

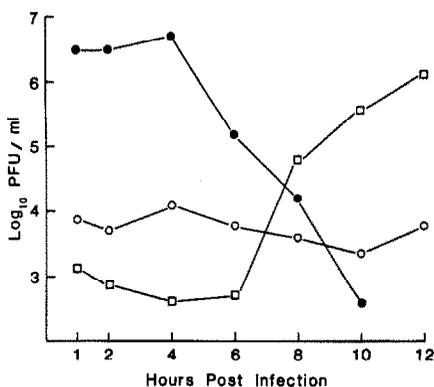


FIG. 3. The growth of JHM virus (□) and S-1JHM virus (○) at 32° and the yield of S-1JHM virus at 12 hr post infection and following shift to the permissive temperature (39°) (●) at the indicated times.

interpret the drop in virus titer occurring between the 8- and 10-hr shift points to reflect inactivation or reabsorption in the absence of new virus synthesis. Similar data were obtained with S-3JHM virus (data not shown), and the yields of JHM virus were not affected by the temperature shifts.

#### Complementation Tests

To determine if S-1JHM virus and S-3JHM virus possessed defects in separate gene products, coinfections of DBT cells with the original S-1JHM and S-3JHM virus pools and the 15 subclones were carried out to detect possible complementation at the restrictive temperature. DBT cells were infected at an m.o.i. of 10.0 and then incubated at the nonpermissive temperature (32°). S-1JHM virus, and all 15 subclones were tested for complementation by coinfection; however, no complementation was detected. The average complementation index (CI) was 0.69. The highest CI value was 1.34 while the lowest was 0.29. Only 8 of the 37 combinations yielded CI values in excess of 1.00. The subclones *cs11*, *cs12*, *cs31*, and *cs34* in combination were also tested for complementation in 17CL1, L-2, and N<sub>2</sub>A cells without an increase in the CI values. In addition to finding improved complementation in different host cells, Gimenez and Pringle (1978) have recently reported improved complementation by increasing the time of adsorption. To test

this, *cs11*, *cs12*, *cs31*, and *cs34* were tested for complementation in DBT cells following 3-hr adsorption at 39°. This time was chosen since the data in Fig. 2 show that the *cs* virus shifted to the nonpermissive temperature for up to 4 hr after infection at the permissive temperature gave reduced yields of infectious virus at 32°. No complementation was detected after prolonged adsorption.

The amount of released virus from the coinfecting cultures was usually lower than that expected from the sum of the yields of the two viruses alone (CI = 1.0). Preble and Younger (1973) have described *ts* mutants which not only fail to complement each other but also inhibit the replication of parental virus. To determine if S-1JHM virus and S-3JHM virus would interfere with the parent JHM virus, DBT cell cultures were coinfecting with either *cs* mutants *cs11* or *cs31* and a large plaque variant of JHM virus (Lai and Stohlman, 1978). The *cs* mutants produce plaques of 4–6 mm while the large plaque size variant of JHM virus produces plaques of 8–11 mm when assayed at 96 hr postinfection, allowing distinction between the two populations. Coinfecting cultures were incubated at the restrictive temperature, and virus was harvested at two time points in separate experiments so different ratios of released *cs* mutant to parental virus could be compared. The virus released from each of five individual plates was titrated. Coinfection resulted in slight but not significant decreases in the yields of both mutant and parental virus at the nonpermissive temperature. A fourfold decrease was found in the yield of *cs31* from cultures coinfecting with parental virus while only a twofold decrease in parental virus was found. Similar experiments carried out at 39° could not be interpreted because an overabundance of *cs* mutant plaques obscured the larger JHM virus plaques.

#### DISCUSSION

Many viruses isolated from persistently infected cell cultures possess properties which differ from those of the parent virus (Preble and Younger, 1973; Rima and

Martin, 1976; Walker, 1964). The virus released from the parental N<sub>1</sub> cells chronically infected with the JHM strain of murine hepatitis virus was, however, identical to the parental virus (Stohlman and Weiner, 1978). It is now apparent that the culture also contained at least two populations of cells which were latently infected with *cs* mutants of JHM virus. The viruses rescued by cell fusion have the same host cell range as JHM virus, produce CPE identical to that produced by the parent JHM virus, and were serologically indistinguishable from JHM virus (Stohlman *et al.*, 1979). We have previously shown that JHM virus shows no growth-related temperature sensitivity over the range of 32 to 39° (Stohlman and Weiner, 1978), and since the isolates are restricted at 32°, we have designated them as *cs* mutants. In addition to being *cs* mutants, both were also more thermally stable than the parent virus. Hence, it appears that the N<sub>1</sub> culture contained cells latently infected with *cs* thermostable mutants of JHM virus. These mutants were probably not released into the supernatant since they were not detected prior to antiviral antibody treatment of the culture (Stohlman and Weiner, 1978).

The rescue of *cs* mutants from these clones was unexpected for several reasons. First, few examples of well-characterized *cs* mutants of animal viruses have been reported (Sommers and Kit, 1973; Wright and Cooper, 1973) although *cs* mutants of bacteriophage are as useful as *ts* mutants in determining gene order (Jarvik and Botstein, 1973). Second, many examples of selection for *ts* mutants during persistent infection have been described (Preble and Younger, 1973; Rima and Martin, 1976; Walker, 1964), but to our knowledge, no example of a *cs* mutant derived from a persistently infected cell line has been reported.

Our results showing that infectious virus can be recovered from cells latently infected with JHM strain of MHV suggest that the genome of a positive stranded RNA virus (Lai and Stohlman, 1978) can persist in cells. Furthermore, the cells need not express detectable viral antigen or contain complete virions or viral subunits demonstrable at the ultramicroscopic level to contain a viral genome capable of being

rescued in the form of infectious virus. Although previous studies of latently infected cells selected by antiviral antibody treatment suggested that the viral genome is propagated by cell division (Rustigian, 1966), we are unable to define a mechanism by which the JHM viral genome replicates and is passaged to the daughter cells. It would appear, however, that JHM virus gene expression can be suppressed at least at two points since one cell clone had detectable viral antigen while the other clone did not. In addition, a complete, albeit altered, genome was present in both cell clones since fusion to permissive cells effected the rescue of infectious virus. Although it may be premature to speculate on the nature of the *cs* defect, it is possible that it resides in a viral-specific RNA polymerase. No polymerase activity has yet been reported in MHV-infected cells, it seems; however, reasonable that this would represent one function expressed prior to the initiation of RNA synthesis.

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