Growth and Maturation of a Vesicular Stomatitis Virus Temperature-Sensitive Mutant and Its Central Nervous System Isolate†

JOSEPH V. HUGHES,1, * TERRY C. JOHNSON,1 STANLEY G. RABINOWITZ,2 AND MAURO C. DAL CANTO3

Division of Biology, Kansas State University, Manhattan, Kansas 66506,1 and Departments of Medicine2 and Pathology (Neuropathology)3, Northwestern University Medical School and Veterans Administration Lakeside Hospital, Chicago, Illinois 60611

Received for publication 5 July 1978

A temperature-sensitive (ts) mutant of vesicular stomatitis virus (VSV), tsG31, produces a prolonged central nervous system disease in mice with pathological features similar to those of slow viral diseases. tsG31 and the subsequent virus recovered from the central nervous system (tsG31BP) of mice infected with tsG31 were compared with the parental wild-type (WT) VSV for plaque morphology, growth kinetics, thermal sensitivity of the virions, and viral protein synthesis and maturation. Several properties of the central nervous system isolate distinguished this virus from the original tsG31 and the WT VSV. The WT VSV produced clear plaques with complete cell lysis, and the tsG31 produced diffuse plaques and incomplete cell lysis, whereas the tsG31BP had clear plaques similar to those of the WT VSV. Although plaque morphology suggested that tsG31BP virus was a revertant to the WT, growth kinetics in either BHK-21 or neuroblastoma (N-18) cells indicated that this virus was similar to tsG31, with a productive cycle at 31°C and no infectious virus at 39°C. At 37°C, however, the tsG31BP matured much slower than did the original tsG31 (and produced only 1% of the yield measured at 31°C). WT VSV produced similar quantities of infectious virions at 31, 37, and 39°C. The lack of infectious virions at 39°C for the ts mutants was presumably not due to a greater rate of inactivation at 39°C. Unlike WT VSV, which synthesized viral proteins equally well at all three temperatures, tsG31 had a reduced synthesis of all the structural proteins at 37 and 39°C, compared with that at 31°C; the formation of the M protein was most temperature sensitive. In addition, fractionation of the infected cells indicated that the incorporation of the M and N proteins into the cellular membranes was also disrupted at the higher, nonpermissive temperatures. Several characteristics of protein synthesis during tsG31BP infection at 39°C distinguished this virus from tsG31: (i) no mature viral proteins were detected at 39°C; (ii) several host proteins were synthesized, suggesting that the virus was incapable of completely depressing host macromolecular synthesis; and (iii) a great proportion of the incorporated radioactivity was found in unusually high-molecular-weight proteins. In addition, at 37°C, the tsG31BP virus showed a decreased synthesis of viral proteins and reduced assembly of the viral structural proteins.

We have recently reported that some temperature-sensitive (ts) mutants of vesicular stomatitis virus (VSV) can alter the usually rapid central nervous system (CNS) disease produced by wild-type (WT) VSV infection of mice (1, 2, 14–16). In particular, mice infected with tsG31 have a more slowly progressive CNS infection, characterized by hind-limb paralysis and death 6 to 9 days after infection (1, 14, 15). In addition, the tsG31 infection was found to be associated with extensive spongiform changes in the gray matter of the spinal cord (1, 14), whereas infection with WT VSV resulted in only minimal changes of encephalitis. tsG31 has been identified as a member of complementation group III (12), which is associated with decreased accumulation and/or functioning of the membrane (M) protein of VSV (7, 8, 10, 13). However, the biochemical nature of the genetic lesion for tsG31 itself has not been described. To better understand the ts lesion in tsG31 which may be

† Contribution no. 78-396-j, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506.
responsible for the altered CNS disease, we compared the tsG31 virus with the WT VSV, with regard to growth and synthesis of viral proteins at permissive and nonpermissive temperatures in tissue culture cells for which experimental conditions could be readily controlled. In addition, we studied the properties of the VSV which was recovered from the CNS of the infected mice (after tsG31 infection). The data in the present report indicate that the CNS isolate (tsG31BP) is quite different from the original tsG31 and, in fact, appears to be more defective during infection at the nonpermissive temperature.

MATERIALS AND METHODS

Cell culture lines. BHK-21 cells, obtained from International Scientific Industries, Cary, Ill., were grown as previously described (14). Neuroblastoma cells, N-18, obtained from Brian Spooner (Division of Biology, Kansas State University, Manhattan), were grown in Nutrient Mixture F-12 (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) supplemented with 10% fetal calf serum (virus-screened; GIBCO), 100 U of penicillin, 100 μg of streptomycin, 2.5 μg of amphotericin B, and 2x amino acid mixture. Both cell lines were grown at 37°C in a 5% CO₂–95% air atmosphere in a water-jacketed incubator.

Viruses. Indiana strains WT VSV and tsG31 were each plaque purified and doubly cloned as previously described (14). Outbred Swiss mice were infected with tsG31, and the virus was isolated 4 days after infection from the brain (tsG31BP) or from the spinal cord (tsG31SC), as described previously (15). The tsG31BP and tsG31SC viruses were plaque purified and doubly cloned.

Plaque assays. BHK-21 cells (5 × 10⁶ to 1 × 10⁶ per ml) were cultured in six-well plates (35 by 10 mm; Linbro Co., New Haven, Conn.) in 2 ml of BHK medium (14). Confluent monolayers were infected by adding 0.1 ml of 10-fold dilutions of freshly thawed virus stock to each series of wells. Virus was allowed to adsorb for 60 min at 31°C, and the wells were overlaid with 2 ml of BHK medium with agar added to 0.37% (Difco Laboratories, Detroit, Mich.). Virus samples were then grown at 31°C in a 5% CO₂–95% air atmosphere. After incubation for 48 h, virus plaques were visualized by staining the monolayers with crystal violet (4).

Growth of the viruses in BHK-21 and N-18 cells. Either BHK-21 or N-18 cells, at a density of 2 × 10⁶ to 4 × 10⁶/ml, were infected at a multiplicity of infection (MOI) of 10. The virus was allowed to adsorb to the cells for 30 min at 25°C. The cells were washed with 10 ml of Hanks balanced salt solution (HBSS) (3) and pelleted by low-speed centrifugation. The cells were washed twice more with HBSS and then diluted to 1 × 10⁶ to 2 × 10⁶ cells/ml. Sealed tubes with 1 × 10⁶ to 2 × 10⁶ cells were incubated as suspensions at 31, 37, or 39°C in water baths. The water baths were maintained at their respective temperatures with a variation of ±0.1°C throughout the culture period. At 1- or 2-h intervals, tubes were removed and frozen at −85°C until the PFU could be determined by using BHK-21 monolayers incubated at 31°C.

Infecting and labeling cell cultures. Either BHK-21 or N-18 cells (usually 3 × 10⁶ cells) were infected with an MOI of 10 to 20 PFU/cell in a small volume (0.5 ml) of HBSS. Actinomycin D (Sigma Chemical Co.) was added to a final concentration of 5 μg/ml for BHK-21 cells and to 20 μg/ml for N-18 cells. Virus was allowed to adsorb for 30 min at 25°C; it was then diluted to 5 ml with labeling medium consisting of HBSS supplemented with minimal essential medium vitamins (GIBCO), 100 U of penicillin, 100 μg of streptomycin, 2.5 μg of amphotericin B, 2 mM L-glutamine, and basal minimal essential amino acids minus methionine. Infected cells were labeled at various times after infection, as indicated in the text, with 6 to 10 μCi of [³⁵S]methionine (New England Nuclear Corp., 400 to 600 Ci/mmol) per ml for a labeling period of 2 h. The cells were then washed twice with HBSS and suspended in saline; Nonidet-P-40 (Shell Oil Co.) was added to a final concentration of 1% to lyse the cells. Nuclei were removed by centrifugation at 1,000 × g for 5 min. The proteins were concentrated from the supernatant by adding 9 volumes of acetone (8).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins. Labeled viral proteins were subjected to gel electrophoresis by the method of Laemmli (9) in a 9% polyacrylamide separating gel (10 cm) and a 3% stacking gel (2 cm). The gels were subjected to electrophoresis at 2.5 mA/gel for approximately 5 h. After electrophoresis, the gels were frozen and sliced (1-mm slices); the gel slices were solubilized in an NCS solution (Amersham Corp., Arlington Heights, Ill.) by mixing overnight. The NCS solution contained 1 volume of NCS to 10 volumes of scintillation cocktail with 4 g of 2,5 diphenyloxazole and 0.06 g of 1,4-bis[2-(4-methyl-5-phenylazo)yl]-benzene per liter of toluene. The radioactivity in each slice was determined by scintillation spectrophotometer, model 3320, with an efficiency of 47% for [³⁵S]methionine. One gel with standard proteins (bovine serum albumin, 66,000; ovalbumin, 45,000; chymotrypsinogen, 25,000) was run with each electrophoresis experiment; the gel was stained with Coomasie brilliant blue to determine the molecular weights of the radioactive viral proteins synthesized during infection.

Viral assembly. To study the intracellular assembly of the VSV proteins, soluble and particulate cell fractions were prepared by a modification of a previously reported procedure (6). Essentiall, the viral proteins were labeled with radioactive methionine as described above, but after the infected cells had been washed with HBSS, they were allowed to swell at 0°C in reticulocyte standard buffer (RSB; 10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl₂, pH 7.4). The cells were then disrupted with 35 to 40 strokes of a tight-fitting, glass-Teflon Dounce homogenizer, and NaCl was added to 100 mM. Nuclei were removed by centrifugation at 1,000 × g for 5 min, and the supernatant fluid was then fractionated on a membrane and particulate fraction by centrifugation for 70 min at 100,000 × g. These fractions were precipitated with acetone, and the specific viral proteins were analyzed by gel electrophoresis as described above.
RESULTS

Plaque morphology. When tsG31 was plaqued on BHK-21 cells, this virus produced a plaque morphology characterized as diffuse with incomplete morphology on light microscopy (Fig. 1B). Observation by light microscopy indicated that many of the BHK-21 cells retained their normal morphology without becoming rounded, as was observed for the WT VSV. The WT VSV produced clear plaques, with almost complete cell lysis (Fig. 1A). Surprisingly, the VSV recovered (4 days after infection) from the brain or spinal cord of Swiss albino mice injected with tsG31 (i.e., tsG31BP or tsG31SC) produced clear plaques similar to those of the WT VSV (Fig. 1C). Such altered plaque morphology suggested that the CNS isolates might be revertants to the WT VSV. To test that possibility, we measured the growth kinetics and protein synthesis by original tsG31, tsG31BP, and the parental WT VSV at 31, 37, and 39°C.

Growth of viruses in BHK-21 and N-18 cells. WT VSV grew well at all three temperatures in both BHK-21 and N-18 cells (Fig. 2), although 20 to 50% more infectious virus was produced at 37°C than at either 31 or 39°C. At all temperatures, growth of WT VSV could usually be detected 4 to 6 h postinfection (p.i.). The yield of virus from N-18 cells was only slightly greater than that from BHK-21 cells. In contrast, when either cell line was infected with tsG31 and incubated at 39°C, there was no detectable production of infectious virions during a 24-h period (Fig. 3). The initial growth of tsG31 at 31 or 37°C was very similar, yet the final yields were much higher at 31°C with the final yield at 37°C being about 10% of that produced at 31°C. As with WT VSV, the N-18 cells yielded more infectious virus, and virus replication was initially detected 4 to 6 h p.i.; it peaked 8 to 10 h p.i.

The growth of tsG31BP (Fig. 4) was similar to that of the original tsG31 with no production of virions at 39°C. Incubation at 31°C resulted in a large yield which began 6 to 8 h p.i. and peaked 12 to 14 h p.i. However, the growth at 37°C for this CNS isolate was quite distinct from either

![Plaque morphology of WT VSV (A), tsG31 (B), and tsG31BP (C) viruses. The viruses were each plaqued on a monolayer of BHK-21 cells for 2 days at 31°C. The plaques were visualized by crystal violet staining of the cells, as indicated in the text.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/on December 18, 2017 by guest)
the tsG31 or the WT VSV. The appearance of infectious virions was not detectable in N-18 cells until 10 h, nor in BHK-21 until 12 to 14 h p.i. The yield at 37°C was usually 1 to 2% of that obtained at 31°C. Similar growth kinetics were observed for the tsG31SC isolate (data not shown).

Since it appeared reasonable that the yield of infectious virions at 39°C might reflect a differential thermal sensitivity of the mutant viruses, the kinetics of thermal inactivation at 39°C was studied. The WT and tsG31 viruses were equally sensitive to incubation at 39°C; tsG31BP virus demonstrated a slightly faster rate of inactivation which was not significantly different from the other viruses (data not shown). Thus, the lack of viral production at 39°C for tsG31 and tsG31BP cannot be explained by a differential sensitivity to thermal denaturation. Presumably, some intracellular block exists at the level of synthesis or maturation of infectious virions at elevated temperatures.

**Synthesis of viral proteins.** As expected from our study of the kinetics of replication of WT VSV, this virus was able to synthesize significant quantities of viral proteins as early as 3 to 5 h p.i. at any of three temperatures tested (31, 37, or 39°C) in either BHK-21 or N-18 cells. The three major structural proteins (N, G, and M) were readily resolved by SDS-PAGE; the L and NS proteins, which are always present in small amounts, were often difficult to detect. In addition, the WT VSV was quite capable of augmenting the action of actinomycin D in inhibiting host protein synthesis, because no other significant amounts of nonviral radioactive proteins were observed by PAGE (data not shown). Although significant amounts of viral proteins were synthesized at all three temperatures tested, more viral proteins accumulated during the 2-h labeling period at 37 and 39°C as compared to 31°C (Table 1). Surprisingly, the synthesis at 39°C was greater than at the other temperatures, even though this was not the most efficient temperature for production of virions (Fig. 2).

In contrast, the synthesis of viral proteins by tsG31 was clearly depressed at high temperatures. Our initial experiments, in which we used a labeling period of 3 to 5 h p.i., showed that no
FIG. 4. Growth of tsG31BP in BHK-21 and N-18 cells. Either BHK-21 (A) or N-18 (B) cells were infected with tsG31BP at an MOI of 10, and the growth kinetics of this virus were determined as described in the legend to Fig. 2.

Viral proteins were apparently synthesized at 39°C in either cell line. By labeling at 5 to 7 h p.i., however, we were able to show that tsG31 virus synthesized viral proteins at all three temperatures (Fig. 5). An analysis of the relative amounts of the three major structural proteins indicated that, unlike WT VSV, there was a significant reduction in viral protein synthesis at both 37 and 39°C (Table 1). The synthesis of all the viral proteins of tsG31 was reduced at these temperatures, although the synthesis and/or accumulation of the M protein was most affected (Table 1). The M protein was not reduced sufficiently to explain the lack of infectious virions at 39°C. Comparing the amount of M protein made at 37 and 39°C by tsG31 showed that the quantities were quite similar although the yield of infectious virions was significantly different (Fig. 3).

Another feature of tsG31 infection at 39°C was a partial deficiency in its ability to augment the inhibitory effects of actinomycin D on host protein synthesis (Fig. 5C). The inability to depress host protein synthesis was detected in both
VOL. 29, 1979
VSV ts MUTANT AND ITS CNS ISOLATE 317

TABLE 1. Viral protein synthesis by WT VSV and tsG31 as a percentage of production at 31°C

<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein</th>
<th>% Synthesized at 37°C</th>
<th>% Synthesized at 39°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT VSV</td>
<td>G</td>
<td>125.9</td>
<td>147.6</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>129.1</td>
<td>140.1</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>125.3</td>
<td>117.7</td>
</tr>
<tr>
<td>tsG31</td>
<td>G</td>
<td>57.7</td>
<td>33.2</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>44.9</td>
<td>37.9</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>22.0</td>
<td>19.7</td>
</tr>
</tbody>
</table>

* The synthesis of the major structural viral proteins at various temperatures was compared by determining the amount of each protein made at each temperature by the individual viruses as resolved by PAGE (as in Fig. 5). For these experiments, viral proteins were labeled in infected N-18 cells during the period of maximal viral synthesis, i.e. 3 to 5 h p.i. for WT VSV and 5 to 7 h p.i. for tsG31. The results for the synthesis at 37 and 39°C are expressed as a percentage of the synthesis at 31°C.

N-18 and BHK-21 cells, even though the concentration of actinomycin D had been increased to 20 μg/ml.

The infection of either cell line with tsG31BP revealed several properties to distinguish this virus from either the original tsG31 mutant or the WT VSV. Interestingly, the tsG31BP virus was quite deficient in its ability to depress host protein synthesis, particularly at 39°C (Fig. 6). In addition, at the nonpermissive temperature the virus did not appear to be capable of synthesizing and/or accumulating a significant amount of viral proteins. At least, no distinct viral radioactive peaks could be distinguished, since many of the protein peaks detected with PAGE were aligned with cellular proteins. At 37°C, at which a lag occurred in the replication (Fig. 4), only very small quantities of viral proteins were detected at 5 to 7 h p.i.; the highest level of accumulation occurred 8 to 10 h p.i. At 31°C, the synthesis of viral proteins was not detected until at least 3 to 5 h p.i.; and again the greatest accumulation of viral polypeptides occurred from 8 to 10 h p.i. Comparing the quantities of viral proteins synthesized at 31 and 37°C indicated that all viral proteins were significantly reduced at 37°C, but the M protein was most affected and accumulated only to 25% of the level attained at 31°C. The reduced production of the structural proteins, particularly M, and the delay in their synthesis, could explain the prolonged and rather sparse replication of tsG31BP at 37°C (Fig. 4).

Another very interesting feature of the proteins made during the infection at high temperatures with tsG31BP was the high-molecular-weight material which migrated to the middle of the stacking gel. Although it is uncertain whether this material was of viral or cellular origin, these labeled polypeptides were present in greater amounts in cells infected at the higher temperatures (37 or 39°C), and greater amounts were synthesized (or accumulated) late in infection. The high-molecular-weight material was not detected in cells infected with WT VSV or in uninfected cells.

Assembly of virions. Since recent reports have indicated that the VSV mutants of complementation group III are defective in the normal processing or maturation of the structural proteins at the nonpermissive temperatures (7), we measured the intracellular distribution of the VSV proteins. A simple fractionation of the infected cells yielded a soluble cytosol fraction and a particulate fraction (100,000 × g pellet) containing both the cell membranes and the nucleocapsids. To reduce the nonspecific or relatively loose association of the M or N proteins with the particulate fraction, 100 mM NaCl was added (7). For experiments with tsG31 and tsG31BP, only a small proportion (less than 2%) of the acid-insoluble radioactivity was recovered from the media in a pellet after centrifugation at 100,000 × g; that indicated that very little of the [35S]methionine was present in mature virions during this experimental procedure. Consequently, only the cellular particulate and cytosol fractions typically were analyzed by PAGE. When this experiment was performed with WT VSV, almost all of the major structural proteins (G, N, M) of the virus were found to be associated with the particulate fraction or with extracellular virus regardless of the temperature tested (data not shown). Similar to previous reports (5, 6, 13), this association was the result of viral proteins rapidly associating with either the cell membrane or with the viral nucleocapsid. In contrast with the WT VSV, the ts mutants demonstrated an assembly process that was highly sensitive to the temperature of incubation.

Surprisingly, the maturation of both the M and N proteins of tsG31 appeared to be blocked at 39°C in N-18 cells (Table 2). There was a much higher percentage of these two structural proteins remaining in the cytosol fraction after the 2-h labeling period. This suggested that the viral proteins of tsG31 at 39°C were incapable of normal assembly. However, there did not appear to be any alteration of the maturation and assembly of the viral proteins at 37°C, since the measurements for the distribution of viral structural proteins at this temperature were very similar to the values seen for WT VSV maturation; however, it should be noted that with tsG31 the amount of M protein was reduced by 78% when infected cells were incubated at 37°C
**Table 2. Maturation of viral proteins**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Incubation temp (°C)</th>
<th>Protein</th>
<th>Cytosol fraction</th>
<th>Particulate fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>tsG31</td>
<td>31</td>
<td>G</td>
<td>1.1</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>45.9</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>G</td>
<td>4.9</td>
<td>95.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>18.7</td>
<td>81.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>21.7</td>
<td>78.3</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>G</td>
<td>2.0</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>55.9</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>61.6</td>
<td>38.4</td>
</tr>
<tr>
<td>tsG31BP</td>
<td>31</td>
<td>G</td>
<td>25.6</td>
<td>74.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>26.9</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>49.5</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>G</td>
<td>24.4</td>
<td>75.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>58.2</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>56.7</td>
<td>43.3</td>
</tr>
</tbody>
</table>

*The percentage of viral proteins present either in the cytosol fractions or in the particulate fraction (100,000 × g pellet) after labeling viral proteins from 5 to 7 h p.i. with [35S]methionine. The fractions were prepared as described in the text, and the amount of each structural protein synthesized was determined by PAGE.*

(The Table 1). Therefore, the amount of M protein associated with the particulate fraction at 31°C was over three times that measured at 37°C. In contrast to the M protein, the G protein was found almost exclusively associated with the particulate fraction at all three temperatures.

The fractionation of N-18 cells infected with tsG31BP suggested that this virus was even more deficient in its assembly than was the original tsG31. Since there did not appear to be any significant accumulation of viral proteins at 39°C, the fractionation of the cells was carried out only with cells incubated at 37 and 31°C (Table 2). Interestingly, the processing of the tsG31BP proteins revealed numerous differences between the original tsG31 and tsG31BP; (i) a smaller portion of the G protein associated with the particulate fraction in the tsG31BP-infected cells at either temperature; and (ii) the N protein appeared to associate mostly with the membrane or nucleocapsid fraction at 31°C (similar to tsG31), yet at 37°C about 60% of the N protein remained in the cytosol. The M protein appeared to be about evenly distributed between particulate and cytosol fractions at both temperatures.

**DISCUSSION**

In previous studies, we described a unique CNS disease in mice that results from intracerebral inoculation with tsG31 and which is quite distinct from the infectious disease caused by WT VSV. WT VSV produces a fulminating CNS infection, with death of the animals within 2 to 3 days (2, 14, 16). Histopathologically, the animals exhibit minimal changes in the CNS, and most of the alterations appear to be limited to moderate encephalitis (14). In contrast to WT VSV infection, the injection of tsG31 produces a more slowly progressive disease, characterized by hind-limb paralysis and death at 6 to 9 days after intracerebral inoculation. Unlike the WT VSV infection, animals inoculated with tsG31 exhibit a marked and extensive status spongiosus in the gray matter of the spinal cord (1, 14, 15). Because such an extensive spongiform myelopathy only rarely has been reported in association with infectious diseases of the CNS, we attempted to characterize the biochemical nature of the original tsG31 mutant and the virus subsequently isolated from the CNS of infected animals (tsG31BP).

The original report that describes the isolation of tsG31 placed this mutant as a member of the genetic complementation group III (12). The temperature sensitivity of other mutants in this complementation group has been primarily associated with a decreased synthesis and/or maturation of the viral M protein (7, 8, 10, 13). Consistent with this classification, our study showed that the synthesis or accumulation of the M protein was markedly reduced when infected cells were infected with tsG31 and incubated at 37 or 39°C (Table 1). In addition, we found that the ability of the M protein to participate in virion assembly at the cell surface (as **Fig. 5. (left)** Viral proteins of tsG31 synthesized in N-18 cells at different temperatures. N-18 cells (2 × 10⁶) were infected at 25°C with tsG31 at an MOI of 10, and then incubated at 31°C (A), 37°C (B), or 39°C (C). At 5 h p.i., [35S]methionine was added, and the cells were reincubated at the initial incubation temperature. At 7 h p.i., the cells were washed and then lysed in reticulocyte standard buffer with 1% NP-40. The nuclei were pelleted by centrifugation, and the remaining cellular proteins were precipitated with 9 volumes of acetone. The proteins were resolubilized in reticulocyte standard buffer and subjected to electrophoresis on a disk gel with a 3% polyacrylamide stacking layer (fractions 1 to 20) and a 9% separating layer (fractions 21 to 110). The gels were sliced (1 mm each) and solubilized in NCS; radioactivity was determined in a Packard TriCarb scintillation counter as described in the text.

**Fig. 6. (right)** Viral proteins of tsG31BP synthesized in N-18 cells at different temperatures. The synthesis of proteins in N-18 cells infected with tsG31BP was assayed as described in the legend to Fig. 5.
measured by its association with the particulate fraction of the cell) was reduced at 39°C (Table 2). The reduced synthesis and maturation of the M protein at 39°C was most likely responsible for the lack of infectious virus yields at 39°C in either BHK-21 or N-18 cells (Fig. 3). At 37°C, despite a similar intracellular level of M protein as measured at 39°C, a significant amount of infectious virus was produced (Fig. 3). That, apparently, was related to the observation that more than 78% of the M protein matured to the cell particulate fraction at this temperature (Table 2). Therefore, the inability of tsG31 to replicate successfully at 39°C was a reflection of both a temperature-sensitive reduction in M protein synthesis as well as its subsequent association with cell membranes as an integral step in virion assembly. The lack of virion assembly at 39°C was also illustrated by the reduced association of the N protein with the infected-cell particulate fractions. Although 90% of the tsG31 N protein was associated with the particulate fraction at 31°C and more than 81% at 37°C, approximately 44% was associated with this fraction at 39°C. That is consistent with the report of Knipe et al. (7), which suggested that the M protein may play a central role in the binding of the N protein at the cell surface during VSV assembly. Despite the primary temperature-sensitive effects on M protein synthesis and maturation, it should be noted that a reduced synthesis of other tsG31 components was also observed at 37 and 39°C (Table 1, Fig. 5). We do not know at present whether or not the accumulation of the G and N proteins was reduced as a consequence of the decreased M protein synthesis or stability at these temperatures.

The virus that we recovered from the CNS of mice infected with tsG31 was quite different from the original mutant. Several characteristics of the tsG31 and the tsG31BP clonal isolates differentiated these viruses. They differed in plaque morphology, growth kinetics at 37°C, and viral protein synthesis at 39°C. Despite the similarity in plaque morphology between the tsG31BP and the WT VSV (Fig. 1), the CNS isolate was clearly not a revertant to the wild type. The tsG31BP virus was quite temperature sensitive in its growth characteristics (Fig. 4), and reintroduction to the CNS of mice produced the prolonged disease and status spongiosus characteristic of the original tsG31 mutant. Similar to tsG31, the reduced growth of the CNS isolate at 37°C appeared to be related to a decreased synthesis of the M protein (at 37°C, there was only 25% of the yield at 31°C). At 39°C, however, there did not appear to be any viral proteins synthesized in cells infected with the CNS isolate. In addition, tsG31BP-infected cells accumulated unusually high-molecular-weight proteins. These high-molecular-weight proteins were not observed at 39°C in either cells infected with WT VSV-uninfected cells or cells prelabeled with [35S]methionine and then infected with tsG31BP virus. In addition, these proteins were not evident at 31°C in uninfected cells, nor in cells infected with WT VSV, tsG31, or tsG31BP. These results suggest that the tsG31BP virus may have a block in the normal synthesis and/or processing of its mRNA (11) at elevated temperatures. This matter should be resolved because it is somewhat complicated by the reduced capacity of tsG31BP to augment the inhibitory activity of actinomycin D at 37 and 39°C (Fig. 6). Why tsG31BP has a reduced ability to inhibit host cell protein synthesis at semipermissive and nonpermissive temperatures is currently unknown, but it may be related to the host-virus interaction at the level of transcription (17).

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant NS 13045 from the National Institute of Neurological and Communicative Disorders and Stroke and grant MR57319 from the Veterans Administration. S.G.R. is a Clinical Investigator of the Veterans Administration.

LITERATURE CITED

VSV ts MUTANT AND ITS CNS ISOLATE


