RNA Degradation Defect in Central Nervous System Isolates of Vesicular Stomatitis Virus

By JOSEPH V. HUGHES1* and TERRY C. JOHNSON2

1Department of Microbiology, University of Kansas Medical Center, 39th & Rainbow Blvd., Kansas City, Kansas 66103, U.S.A. and 2Division of Biology, Kansas State University, Manhattan, Kansas 66506, U.S.A.

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SUMMARY

Six temperature-sensitive (ts) mutants of vesicular stomatitis virus (VSV) isolated from the central nervous system (CNS) following injection with ts G31 (III) all possessed a post-transcriptional defect, not found in the initial virus, that affects the stability of viral RNA transcripts. Examination of viral RNA metabolism in mouse neuroblastoma (N-18) cells revealed that RNA synthesis of the CNS isolates was decreased considerably at elevated temperatures (up to 80 or 90% at 39 °C). In addition, analysis of the RNA transcripts suggested that little if any normal-sized transcripts were made in cells infected with these CNS isolates at either 37 °C or 39 °C. The RNA deficiencies did not appear to be the result of a temperature-sensitive lability of virion transcriptase as examined by in vitro transcriptase assays. However, when N-18 cells infected with one of the CNS isolates, ts G31 BP, were first preincubated at the permissive temperature of 31 °C for 3 h and then shifted to 39 °C, RNA synthesis proceeded at a rate comparable to that of 31 °C. The viral mRNA species synthesized following the temperature shift also contained normal-sized tracts of poly(A) RNA, suggesting that neither the viral transcriptase nor its polyadenylate synthetase was thermally labile. However, for any of the six CNS isolates, all species of viral RNA synthesized in cells that were first preincubated at 31 °C degraded rapidly when the cells were shifted to 39 °C. In contrast little or no RNA degradation of either 42S progeny RNA or mRNA species was detected in the wild-type VSV, ts G31 or three other VSV mutants that are defective in some aspect of viral RNA metabolism: [ts G11 (I), ts G22 (II), ts G41 (IV)]. The apparent phenotype alteration in the stability of viral RNA in all of these CNS isolates is discussed in terms of the possible genotypic changes that may have occurred as well as the unique CNS disease that accompanies infection by these viruses.

INTRODUCTION

The intracerebral injection of a temperature-sensitive (ts) mutant of vesicular stomatitis virus (VSV), i.e. ts G31, results in a unique central nervous system (CNS) disease highlighted by a slow progressive infection, hindlimb paralysis and extensive neuropathological lesions (Dal Canto et al., 1976a, b, 1979; Rabinowitz et al., 1976, 1977b). These features are quite unlike the rather swift wild-type (wt-VSV) VSV infection (Doyle & Holland, 1973; Rabinowitz et al., 1976; Wagner, 1974). The histopathological features, including status spongiosus, of the ts G31 CNS infection are strikingly similar, however, to those of several slow virus diseases such as kuru, Creutzfeldt-Jakob disease and scrapie that appear to be
associated with atypical viral agents (Dal Canto et al., 1976b; Lampert et al., 1969, 1972). In recent studies (Hughes et al., 1979b; Hughes & Johnson, 1980, 1981), we have reported that the only virus recovered from the CNS of mice infected with ts G31 was quite different from the original ts G31 mutant. This CNS isolate, ts G31BP, displayed a temperature sensitivity for growth, protein and RNA synthesis; a different plaque morphology; and altered peptides in the M and N proteins that contrasted to ts G31. It now appears that this ts G31BP virus may in fact be responsible for the slowly progressive course of disease and spongiform myelopathy in the CNS, since reinjection of ts G31BP results in the disease pattern originally ascribed to the ts G31 mutant.

The dissimilarities between ts G31 and ts G31BP prompted us to reexamine whether ts G31 could ever be reisolated from the CNS following intracerebral injection. Although several isolations have been attempted, only virions similar to ts G31BP were obtained from the infected animals. Most surprisingly, all of the CNS isolates examined appear to be temperature-sensitive for growth and particularly for RNA synthesis. In addition, all of the CNS isolates will induce a slow progressive disease with hindlimb paralysis when injected intracerebrally into mice. We report here our studies on the defects in the viral RNA metabolism that underlie the ts defects of several of these altered CNS isolates.

METHODS

Cell culture lines and viruses. BHK-21 cells from International Scientific Industries (Carry, Ill., U.S.A.) and murine neuroblastoma cells, N-18, obtained from Brian Spooner (Division of Biology, Kansas State University, Manhattan, U.S.A.) were grown as previously described (Hughes et al., 1979b). Indiana serotype wt-VSV and ts G31 were each plaque-purified and double cloned as described by Rabinowitz et al. (1976). Virus stocks were grown in BHK-21 cells with a low multiplicity of infection for 1 day of incubation at 37 °C for wt-VSV and 2 days at 31 °C for ts G31, and the virus was purified by sucrose gradient centrifugation from which only the B-type virion was isolated (Rabinowitz et al., 1977a). Outbred Swiss mice were infected with ts G31 and virus was isolated from the brain 4 days after infection and was designated ts G31BP (Hughes et al., 1979b). More CNS isolates were obtained, again using outbred Swiss mice infected with ts G31, and these were designated HJ-A1, -B1, -C1, -D1 and -E1 (J. V. Hughes & T. C. Johnson, unpublished results). All of the CNS isolates were also plaque-purified, double cloned and then virus stocks were grown in BHK-21 cells and purified by sucrose gradient centrifugation.

The following ts mutants, all Glasgow isolates, with their respective complementation group listed in parentheses were obtained from Dr M. E. Reichmann (University of Illinois, Urbana, Ill., U.S.A.) and Dr R. R. Wagner (University of Virginia, Charlottesville, Va., U.S.A.): ts G11 (I), ts G22 (II), ts G31 (III) and ts G41 (IV). Ts G11, ts G22 and ts G41 have all been described previously as RNA mutants and are restricted for RNA accumulation at high temperatures (Pringle & Duncan, 1971; Combard et al., 1974; Hunt & Wagner, 1974). While complementation group I mutants are generally recognized as having a defect in viral transcriptase (L protein) and group III mutants have a defect in the matrix (M) protein, the exact assignment of virus proteins to complementation groups II and IV is still somewhat uncertain. Ts G31BP does not complement with any of the Glasgow isolates listed above.

RNA synthesis. N-18 cells (2 x 10⁶ to 4 x 10⁶ cells) were infected with a multiplicity of 10 to 20 p.f.u./cell and actinomycin D (Sigma) was added to 20 μg/ml; this concentration of actinomycin was maintained throughout the preincubation and labelling periods. After 20 min of adsorption the cells were diluted with labelling media [consisting of Hanks’ balanced salt solution (HBSS) supplemented with minimal essential medium vitamins, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM-L-glutamine, and basal minimal essential amino acids] and then incubated at the appropriate temperatures. Infected
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The cells were then labelled at 2 h after infection for 2 h with the addition of 5 μCi [3H]uridine/ml (14 Ci/mmol; Amersham Corporation). Following incubation, the cells were washed twice with HBSS and macromolecules were precipitated with 10% trichloroacetic acid (TCA). The precipitate was then twice washed by repeated precipitation with 5% TCA and the entire sample was collected and washed on to glass fibre filters with 5% TCA, and counted in 3a70b scintillation fluid (Research Products International, Elk Grove Village, Ill., U.S.A.).

When the newly synthesized virus RNA was analysed by SDS–sucrose gradients, the cells were infected and labelled in the presence of actinomycin D at 20 μg/ml, and then washed with HBSS as above. The cells were lysed in RSB (10 mM-tris–HCl pH 7-4, 10 mM-NaCl, 1·5 mM-MgCl2) containing 1% Nonidet P40 (NP40), bentonite at 2 mg/ml, and heparin at 1000 units/ml. Following cell lysis, the nuclei were removed by slow-speed centrifugation, and the supernatant was washed with 1% SDS and then layered on to a linear 15 to 30% sucrose gradient (in a buffer of 10 mM-NaCl, 1 mM-EDTA, 0·5% SDS, 10 mM-tris–HCl pH 7·4). These were centrifuged for 14 h at 23000 rev/min in a Beckman SW41 rotor (Perlman & Huang, 1973), and mouse brain ribosomal RNA was centrifuged with each experiment to estimate sedimentation values of the newly synthesized RNA. Fractions were collected from the bottom of each gradient and processed by precipitating the RNA with 10% TCA, with yeast RNA (1 mg/ml) added as carrier. The RNA precipitates were pelleted by low-speed centrifugation, solubilized in RSB and 1 M-NaOH, dissolved in Biofluor scintillation fluid (New England Nuclear) and the radioactivity present was determined.

Poly(A) synthesis was determined using [3H]adenine (23·6 Ci/mmol; New England Nuclear) to label the virus RNA in infected N-18 cells. The RNA was then sedimented on SDS–sucrose gradients, as described above; the fractions containing the mRNA species, 28S and 15S to 12S, were collected and precipitated repeatedly with ethanol. The RNA was then dissolved in HSB (300 mM-NaCl, 10 mM-tris–HCl pH 7·6) and digested with ribonuclease A at 50 μg/ml and T1 ribonuclease at 25 units/ml for 30 min at 37 °C (Reichmann et al., 1974). The RNase-resistant RNA was washed by precipitation in ethanol and then subjected to electrophoresis on 10% polyacrylamide gels (McLaughlin et al., 1973).

Transcriptase assay. For the in vitro measurement of the transcriptase activity, fresh stocks of the individual viruses were prepared and each was purified on sucrose gradients and tested for activity on the same day. The reaction mixture (Bishop, 1978) consisted of the virions at 200 to 500 μg protein/ml, 100 mM-NaCl, 50 mM-tris–HCl pH 8, 5 mM-MgCl2, 4 mM-dithiothreitol, 1 mM each of ATP, CTP and GTP, 0·05% Triton X-100 and [3H]UTP (16 Ci/mmol; ICN Pharmaceuticals, Irvine, Ca., U.S.A.) at 10 μCi/ml. Reactions were carried out in small volumes (0·2 ml) incubated at various temperatures, and then the labelled RNA was precipitated with 10% TCA. The precipitates were washed twice in 5% TCA, then collected on Millipore HA filters and washed several times with 5% TCA; the dried filters were counted in 3a70b scintillation fluid.

RESULTS

RNA synthesis by ts G31 and its CNS isolates

Since all of the isolates obtained from the CNS following i.c. injection of ts G31 were temperature-sensitive for growth at high temperatures (J. V. Hughes & T. C. Johnson, unpublished results), we initially attempted to determine whether these virions were also temperature-sensitive for RNA synthesis. All of the CNS isolates demonstrated a significantly reduced level of RNA synthesis at 37 or 39 °C (compared to 31 °C) in infected N-18 cells (Table 1). This was in contrast to the wt-VSV, which demonstrated similar synthesis rates at all temperatures tested and to the original mutant ts G31, which appeared to be stimulated at 37 °C and somewhat decreased at 39 °C.
Fig. 1. SDS-gradient analysis of RNA synthesized in N-18 cells infected with ts G31BP. N-18 cells (4 \times 10^6) were infected with ts G31BP at a multiplicity of 10 and incubated at (a) 31 °C, (b) 37 °C or (c) 39 °C. [\textsuperscript{3}H]uridine (20 \mu Ci/ml) was added 2 h after infection. At 6 h after infection, the cells were washed and lysed, and RNA was isolated as described in the text. The RNA was sedimented on an SDS–sucrose gradient for 14 h at 23000 rev/min in a Beckman SW41 rotor. Fractions were collected and processed by precipitating the RNA with 10% TCA. The RNA was labelled by low-speed centrifugation, solubilized in RSB and 1 M NaOH, dissolved in Biofluor scintillation fluid and the radioactivity present was determined. Sedimentation is from right to left, and mouse brain ribosomal subunit RNA was used as marker RNA (28S and 18S RNA).

Table 1. Virus RNA synthesis

<table>
<thead>
<tr>
<th>Virus</th>
<th>Incorporation of [\textsuperscript{3}H]uridine (ct/min)*</th>
<th>Synthesis ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>wt-VSV</td>
<td>3653</td>
<td>4780</td>
</tr>
<tr>
<td>ts G31</td>
<td>1509</td>
<td>3710</td>
</tr>
<tr>
<td>CNS isolates of ts G31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ts G31BP</td>
<td>1589</td>
<td>526</td>
</tr>
<tr>
<td>HJ-A1</td>
<td>2347</td>
<td>968</td>
</tr>
<tr>
<td>HJ-B1</td>
<td>2711</td>
<td>1439</td>
</tr>
<tr>
<td>HJ-C1</td>
<td>1923</td>
<td>1561</td>
</tr>
<tr>
<td>HJ-D1</td>
<td>1572</td>
<td>1201</td>
</tr>
<tr>
<td>HJ-E1</td>
<td>1789</td>
<td>1043</td>
</tr>
</tbody>
</table>

* N-18 cells were infected with the indicated viruses and incubated at the three temperatures, after which the viral RNA was labelled as described in the text. The values for the incorporated ct/min represent the average of triplicate measurements for RNA synthesized 2 to 4 h after infection.

In order to better measure the extent to which viral RNA synthesis was deficient at high temperatures for these CNS isolates and to identify the RNA species most affected, virus RNA was isolated from infected cells and sedimented on SDS–sucrose gradients. In ts G31BP-infected N-18 cells, only small amounts of RNA were detected at either 37 °C or 39 °C (Fig. 1). At 37 °C the 42S RNA was only 20% of the yield at 31 °C and the mRNA species (28S and 15S to 12S) were reduced to less than 10% of the yield at 31 °C. At 39 °C, even more drastic alterations were seen and no normal-sized virus RNA species accumulated. Only a single peak of low mol. wt. RNA was detected at the top of the gradient. The addition of cycloheximide to cells early after infection (in order to block secondary transcription) did
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Fig. 2. SDS-gradient analysis of RNA synthesized in ts G31-infected N-18 cells. N-18 cells (5 x 10⁶) were infected with ts G31 at a multiplicity of 8 and the viral RNA was labelled and isolated as described in the legend to Fig. 1. (a) 31 °C, (b) 37 °C and (c) 39 °C.

Fig. 3. Transcriptase activity measured at different temperatures. The transcriptase activity of isolated virions (purified on sucrose gradients) was determined as described in the text by incubating the virions of (a) wt-VSV, (b) ts G31 or (c) ts G31BP at either 31 °C (●) or 39 °C (○). The incorporation of [³H]UTP into TCA-precipitated RNA was determined for triplicate samples for 60 and 120 min of incubation.

not result in any greater accumulation of RNA at either 37 °C or 39 °C suggesting the RNA metabolic defect was not primarily limited to steps in secondary transcription as opposed to primary transcription. The other five CNS isolates (HJ-A1, -B1, -C1, -D1 and -E1) all demonstrated very similar results when their newly synthesized viral RNA was analysed on SDS-sucrose gradients, being temperature-sensitive for both virion and mRNA species at both 37 °C and 39 °C.

The temperature sensitivity of these CNS isolates for RNA synthesis contrasted sharply to the synthesis of RNA in N-18 cells infected with ts G31. At 37 °C the levels of all species of
VSV RNA remained high, and were equal to or greater than that measured at 31 °C (Fig. 2). Even at 39 °C both 42S RNA and mRNA could be detected in cells infected with ts G31. Similar experiments with N-18 cells infected with wt-VSV demonstrated that similar amounts of all RNA species were made at 31 °C, 37 °C or 39 °C (data not shown). Further experiments were designed to take a closer look at the deficiency in viral RNA metabolism that might underlie the lack of RNA accumulation for one of the CNS isolates, i.e. ts G31BP.

**Temperature sensitivity of the virion transcriptases**

The lack of viral RNA accumulation in cells infected with any of the CNS isolates at elevated temperatures suggested that these mutants might possess a temperature-sensitive transcriptase activity. In order to measure the thermal sensitivity of RNA transcription for one of these virions, ts G31BP, our initial experiments consisted of incubating isolated virions at different temperatures in an *in vitro* transcriptase assay (Fig. 3). The wt-VSV synthesized RNA at 31 °C for at least 2 h, while at 39 °C the rate and total accumulation of wt-VSV RNA was significantly reduced, with the production of only 15 to 19% of the amount synthesized at 31 °C. The ts G31 and ts G31BP viruses synthesized RNA at 31 °C and 39 °C in a very similar fashion as the wt-VSV. In particular, the synthesis of RNA at 39 °C for ts G31 was 12 to 18% of the 31 °C yield and for ts G31BP was 12 to 17% of the 31 °C yield (Fig. 3). This assay thus suggested that the ts G31BP transcriptase was no more thermally sensitive than the wt-virion transcriptase in this *in vitro* assay.

As there are some difficulties in measuring the viral transcriptase activity at elevated temperatures (Bishop, 1978), we have also measured the thermal stability of these various viral transcriptases. Isolated virions were first incubated at 39 °C for various times and then the heated and unheated (control) virions were tested for their ability to transcribe RNA in the standard transcriptase assay at 31 °C (the optimal temperature for this enzyme assay). Again it appeared that the ts G31BP transcriptase activity was no more heat-labile than the wt-virion transcriptase (Fig. 4). Although not shown, the transcriptase of the original ts G31 was also found to be heat-insensitive. In contrast, ts G11, which was previously shown to have a temperature-sensitive transcriptase enzyme activity (Pringle & Duncan, 1971; Hunt & Wagner, 1974), was more rapidly inactivated by incubation at 39 °C, losing up to 50% of its activity by 45 min (Fig. 4).
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### Table 2. Synthesis of RNA following a temperature shift

<table>
<thead>
<tr>
<th>Reincubation temperature (°C)</th>
<th>42S ct/min</th>
<th>28S ct/min</th>
<th>15S to 12S ct/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>2736</td>
<td>3868</td>
<td>19968</td>
</tr>
<tr>
<td>39</td>
<td>2460</td>
<td>6165</td>
<td>20296</td>
</tr>
</tbody>
</table>

* N-18 cells were infected with *ts* G31BP as described in the text and the cells were then incubated at 31 °C for 3 h. The infected cells were then washed, resuspended in media containing [3H]adenine at 40 μCi/ml, and then reincubated at 31 °C or 39 °C for 60 min. The viral RNA was then isolated as described in Fig. 1 and sedimented on an SDS–sucrose gradient. Fractions were collected from the gradients and the various species of RNA (42S, 28S and 15S to 12S) were pooled prior to determining the amount of radioactivity incorporated in each species. Comparable results were observed in two separate experiments and one experiment utilizing [3H]uridine as the radioactive precursor.

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**Viral RNA and poly(A) RNA synthesis after temperature shift**

Since the *in vitro* assays of the viral transcriptase suggested that the RNA synthetic capabilities of *ts* G31BP were not temperature-sensitive, we examined whether this virus could synthesize virus RNA in infected cells at the non-permissive temperature following a preliminary incubation at 31 °C. *Ts* G31BP-infected cells were incubated at 31 °C for 3 h and then aliquots were reincubated at either 31 °C or 39 °C and [3H]adenine was added to assess the virus' ability to synthesize RNA (Table 2). Under these conditions, the *ts* G31BP virus appeared to be quite capable of synthesizing all of the species of viral RNA at the normally non-permissive temperature of 39 °C. In addition, we have also examined the poly(A) RNA that was attached to the viral mRNA species during the reincubation period. Viral RNA was labelled by the addition of [3H]adenine to the infected cells; mRNA was isolated from the appropriate regions on SDS gradients and then subjected to digestion by RNase A and T1. The RNase-resistant poly(A) RNA was then separated on SDS–polyacrylamide gels. The poly(A) RNA that was synthesized at 39 °C in *ts* G31BP-infected cells was as extensive and approximately the same size as the poly(A) RNA made at 31 °C (Fig. 5). The results of these *in vivo* temperature shift experiments, which are in sharp contrast to the complete lack of viral RNA when cells are incubated continuously at
39 °C, suggested that the ts defect in ts G31BP was not in the RNA synthetic capability of the virus. The RNA deficiencies may have resulted, however, from a rapid degradation of viral RNA at the elevated temperatures.

**Virus RNA stability**

To measure virus RNA stability, infected N-18 cells were incubated at 31 °C and [3H]uridine was added for a 4 h incubation period so that a significant amount of RNA was radiolabelled. The cells were then split into three aliquots, one to determine the amount of RNA synthesized during the 4 h labelling period (Fig. 6a) and the other two for reincubation at either 31 °C or 39 °C (Fig. 6b, c). The amount of RNA in ts G31BP-infected cells reincubated at 39 °C was greatly decreased for all three species of RNA, while reincubation at 31 °C did not result in any significant change in the concentration of radioactive RNA present intracellularly.

Similar experiments were performed to measure RNA degradation for several viruses compiled from a number of SDS-gradient analyses (Table 3). Since the amount of RNA remaining after 60 min of reincubation at 31 °C was essentially the same as for the 0 time aliquot (with no reincubation), Table 3 presents only the ratio of the RNA remaining at 39 °C (after 60 min) to the RNA at 31 °C. The ts G31BP virion showed a decrease of about 28% of its 42S RNA and approx. 45% of its mRNA species (28S and 15S to 12S RNA). This decrease in intracellular RNA appeared to be the result of intracellular degradation, as no labelled RNA was recovered from the extracellular media. In contrast, neither the wt-VSV nor ts G31 had a rapid loss of any of the RNA species, and the ts G31 appeared to be particularly stable after the infected cells had been shifted to 39 °C. We have also examined the other five CNS isolates for their sensitivity to in vivo RNA degradation. Interestingly, all five virions demonstrated a high degree of degradation when the cells infected with the CNS isolates were shifted to 39 °C (Table 3). In fact, some of the CNS isolates, HJ-C1, -D1 and -E1, appear...
**RNA degradation defect in VSV**

Table 3. *RNA remaining after shifting infected cells to 39 °C*

<table>
<thead>
<tr>
<th>Virus</th>
<th>RNA at 39 °C/RNA at 31 °C*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42S</td>
</tr>
<tr>
<td>wt</td>
<td>0.93 (+.01)</td>
</tr>
<tr>
<td><em>ts G31</em></td>
<td>1.06 (+.02)</td>
</tr>
<tr>
<td>CNS isolates of <em>ts G31</em></td>
<td></td>
</tr>
<tr>
<td><em>ts G31BP</em></td>
<td>0.72 (+.07)</td>
</tr>
<tr>
<td>HJ-A1</td>
<td>0.71</td>
</tr>
<tr>
<td>HJ-B1</td>
<td>0.64</td>
</tr>
<tr>
<td>HJ-C1</td>
<td>0.27</td>
</tr>
<tr>
<td>HJ-D1</td>
<td>0.37</td>
</tr>
<tr>
<td>HJ-E1</td>
<td>0.63</td>
</tr>
<tr>
<td>Other VSV mutants</td>
<td></td>
</tr>
<tr>
<td><em>ts G11</em></td>
<td>0.98 (+.03)</td>
</tr>
<tr>
<td><em>ts G22</em></td>
<td>0.97 (+.05)</td>
</tr>
<tr>
<td><em>ts G41</em></td>
<td>0.90 (+.10)</td>
</tr>
</tbody>
</table>

*N-18 cells were infected with the indicated viruses and incubated at 31 °C; [3H]uridine was added to label the viral RNA, and then the infected cells were washed and reincubated as described in Fig. 6. The amount of RNA remaining after 60 min of reincubation at either 31 °C or 39 °C was determined from SDS gradients of the labelled RNA, and the ratio presented here is the amount left at 39 °C compared to the RNA left at 31 °C. The values are the means of two to four independent determinations, with the ranges indicated in parentheses except for the single determinations with HJ-A1, -B1, -C1, -D1 and -E1. The 0 time aliquot did not differ significantly from the aliquot incubated at 31 °C for 60 min for any of these viruses.

...to be much more sensitive than the *ts G31BP* virion to a shift to 39 °C as the majority of their viral RNA species was degraded during the 60 min reincubation at this elevated temperature.

We also examined three other VS virions that have all been described as temperature-sensitive mutants defective in some aspect of RNA metabolism (Combard *et al.*, 1974; Hunt & Wagner, 1974; Pringle & Duncan, 1971). With both *ts G11* (a virus having a heat-labile transcriptase enzyme) and *ts G41*, the viral RNA synthesized at 31 °C remained very stable after the infected cells had been shifted to 39 °C. Only *ts G22* appeared to have a high rate of RNA degradation at 39 °C, although apparently only the mRNA species of 28S and 15S to 12S RNA appeared to be affected and the amount of degradation was less than was detected with any of the CNS isolates.

**DISCUSSION**

The studies described in this paper were designed to characterize more thoroughly the CNS isolates obtained after *ts G31* infection to determine how the virus population changed from the original *ts G31*, as well as to attempt to understand its possible role in the progressive spongioform myelopathy seen in the infected mouse CNS. In contrast to the original *ts G31* virus, all of the brain isolates possessed a defect that affects the stability of newly synthesized viral RNA and prevents accumulation of viral RNA and proteins and virion maturation at non-permissive temperatures (Table 1; Fig. 1; Hughes *et al.*, 1979a, b). Although we initially suspected that the lack of RNA accumulation at 39 °C *in vivo* might be the result of a temperature-sensitive transcriptase enzyme, a number of experiments demonstrated that this was not the case at least for *ts G31BP* (Fig. 3, 4). The *ts G31BP* transcriptase appeared to be as thermally stable as the wt-VSV and the original *ts G31* in these *in vitro* assays. In contrast, *ts G11*, a group I mutant previously described as a defective transcriptase mutant (Hunt & Wagner, 1974), did appear to have a heat-labile transcriptase activity under these conditions. Although not all group I mutants have heat-labile transcriptases, this assay appears to present one of the clearest methods for determining the temperature sensitivity of the VSV transcriptase (Bishop, 1978). *In vitro* transcriptase assays are now being performed with the HJ virions.
Surprisingly, the ts G31BP virus was quite capable of synthesizing normal-sized transcripts following the shift up to 39 °C (Table 2) from the permissive 31 °C temperature. Although we initially thought a defective polyadenylation of viral mRNA could underlie the temperature-sensitive defect for ts G31BP RNA metabolism, it appears that this virus is able to polyadenylate its mRNA species at both 31 °C and 39 °C following a temperature shift (Fig. 5). Therefore, both the in vitro transcriptase assays and the in vivo RNA synthesis experiments following a temperature shift, indicate that the ts G31BP virus probably does not have a temperature-sensitive defect in either transcription or polyadenylation.

Moreover, the rapid degradation of the viral RNA made in ts G31BP-infected cells provided evidence strongly suggesting that newly synthesized virus RNA was very unstable when infected cells were incubated at 39 °C (Fig. 6). The addition of excess unlabelled uridine and glucosamine to the cells during the chase periods in these experiments allowed us to limit any further incorporation of [³H]uridine (Weck & Wagner, 1978; Wertz, 1975), so that we could estimate the rate of RNA degradation alone. Although degradation is not immediate after shifting the cells to 39 °C, the rate is sufficient to degrade 45 to 50% of the RNA made in 240 min by only 60 min. Surprisingly, when the other CNS isolates were examined in this assay, all were quite sensitive to degradation at 37 °C and 39 °C (Table 3). Almost all other VSV mutants including representatives of complementation groups I, III and IV, had thermally stable viral RNA. The exception, ts G22 of group II, did have RNA instability but only of its mRNA species. Thus, surprisingly, all the CNS isolates stand out as possessing unstable mRNA species as well as 42S progeny RNA species. Although a rapid decay of VSV RNA has been noted by other investigators (Pennica et al., 1979), the amount of decay we have seen for these CNS isolates is much faster than was demonstrated in this previous manuscript, and of course is faster than the rate of degradation of wt-VSV, ts G31, etc. (Table 3). One possible explanation for the labile RNA of ts G31BP (the CNS isolate we have most closely examined) could involve a defective viral protein that may not protect the newly made virus RNA. Although it is generally recognized that the N protein binds tightly to and could protect the viral 42S RNA of VSV, there is some conflicting evidence with regard to whether this protein is associated in the cell with the viral mRNA species. Soria et al. (1974) provided evidence that the N protein is associated with both the negative- and positive-stranded 42S RNA, and that the protein did not apparently associate with the mRNA complexes. On the other hand, Grubman & Shafritz (1977) examined VSV proteins on RNA species isolated from polyribosomes and concluded that the VSV N protein was associated with mRNA. Assuming that the N protein may be involved with intracellular stability of either the 42S RNA or both the 42S RNA and the mRNA species, we have been investigating the peptide structure of the N protein in ts G31BP by analysis of protease digestions. Indeed, the N protein of ts G31BP appears to be altered from the ts G31 N protein (Hughes & Johnson, 1981). In a separate study, we have also examined the melting kinetics of isolated viral nucleocapsids, which consist solely of the N protein and viral RNA. These studies demonstrated a much larger hyperchromatic shift upon heating ts G31BP nucleocapsids than was seen with the wt-VSV or ts G31 nucleocapsids (Hughes & Johnson, 1980). These results suggest that the ts G31BP virion possesses a different type of RNA:N protein interaction, presumably much weaker than that of the wt-VSV or ts G31, which may play some role in the apparent lability of newly synthesized RNA species in ts G31BP-infected cells at elevated temperatures. We are presently analysing the proteins and peptide structures of the other CNS isolates. Although it is certainly not clear at this point if all of these isolates will have the same genetic alteration (from the original ts G31 virion) it is most intriguing that all of the initial CNS isolates we have examined possess the very similar phenotype of rapidly degraded virus RNA at elevated temperatures.
RNA degradation defect in VSV

The selection in this CNS infection of mutants defective in RNA metabolism may be similar to previous reports with persistent infections of tissue culture cell lines with VSV (Holland et al., 1976, 1979; Youngner et al., 1976, 1978). First, mutant viruses appear to be selected that are less capable of replication and damage to the host at elevated temperatures during the course of these persistent infections. Second, there appears to be some selection of RNA mutants in particular, although the selection pressure for this is presently unknown (Youngner et al., 1978). As suggested previously (Holland et al., 1976, 1979; Youngner et al., 1978), the selection of mutants that are more defective in their early replication may be essential for maintaining long-term persistent infections as well as necessary for the induction of a more slowly progressive disease such as we have seen in the mouse CNS.

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