JOURNAL OF VIROLOGY, Mar. 1977, p. 1140-1148

Vol. 21, No. 3
Printed in U.S.A.

Analysis of the Defects of Temperature-Sensitive Mutants of Vesicular Stomatitis Virus: Intracellular Degradation of Specific Viral Proteins

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Received for publication 10 September 1976

The metabolism of viral RNA and proteins has been studied in cells infected with temperature-sensitive mutant strains of vesicular stomatitis virus. Certain viral proteins encoded by the mutant strains, usually the putative mutant protein for the assigned complementation group, were shown to be degraded more rapidly at the nonpermissive temperature than were the wild-type proteins. Group III mutants (*ts*G33, *ts*M301) encode M proteins which are degraded three- to fourfold faster than the wild-type protein. This defect cannot be fully rescued by coinfection with wild-type virus, and thus the defect appears to be in the M protein itself. Mutants *ts*M601(VI) and *ts*G41(IV) encode N proteins which are degraded much faster than the wild-type protein and also share the property of being defective in replication of viral RNA, suggesting a correlation between these phenotypic properties. Furthermore, the L proteins of *ts*G11(I) and *ts*G13(I) are more labile than the wild-type protein at the nonpermissive temperature. The G protein of *ts*M501(V) did not undergo the change in electrophoretic mobility previously shown to be the result of sialylation, suggesting that it is defective in maturation or glycosylation at the nonpermissive temperature. Three of the mutants previously isolated in this laboratory, *ts*M502(V), *ts*M601(VI), and *ts*M602(VI), were shown to be defective in viral RNA synthesis at the nonpermissive temperature. Mutant *ts*M601(VI) was defective mainly in viral RNA replication, whereas *ts*M502(V) appeared to be totally defective for viral RNA transcription and replication at the nonpermissive temperature.

Temperature-sensitive mutants of vesicular stomatitis virus (VSV) have been isolated in several laboratories and classified into six complementation groups (2, 3, 6, 18, 19, 21). Group I contains more than one-half of the mutants isolated, and some of these are defective for transcription of mRNA species in vivo at the nonpermissive temperature (15, 16, 20, 23). In addition, reconstitution studies of the transcriptase enzyme showed that the L protein is defective in these mutants (7, 8). Similar reconstitution studies have led to the suggestion that group IV mutations are defective in the N protein (14). Mutants in group III are believed to be defective in the M protein because, in infected cultures shifted from the nonpermissive to permissive temperatures, M protein previously labeled at the nonpermissive temperature cannot be incorporated into virions (11). Group V mutations can be complemented by RNA tumor viruses (24), have been shown to be defective in maturation of the glycoprotein (11), and thus appear to reside in the gene for G protein. No consistent phenotype has been observed for the group II mutants.

In this paper we report on a new property of certain classes of mutants, lability of the putative mutant viral protein at the nonpermissive temperature. In addition, we have observed electrophoretic differences between certain viral proteins of the Glasgow and Massachusetts virus strains. Analysis of the fate of specific mutant gene products in cultures coinfected with two virus strains allowed us to further characterize the nature of the mutations.

MATERIALS AND METHODS

Growth of viruses. Wild-type VSV was prepared as described previously (9). Mutants of the Glasgow series and *ts*O45(V) were obtained from Craig Pringle, Glasgow University. Purified B virions from plaque-purified stocks of these viruses were used in all experiments. Plaque-purified stocks of the Massachusetts mutants were kindly supplied by Carl Rettenmier. Stocks were grown by infecting L-cells at 0.1 PFU/cell and allowing the infection to proceed for 20 h at 31°C (9). To conserve the plaque-purified preparations, these unpurified stocks were used for

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the experiments in this paper. All but two of the mutants [*ts*M502(V) and *ts*M603(VI)] demonstrated wild-type levels of RNA synthesis at the permissive temperature. Labeled viral particles prepared from cultures infected with these two mutants sedimented only as B virions. Thus, there was no evidence of T particle contamination of the preparations.

Measurement of virus-specific RNA accumulation. Viral RNA accumulation was studied as described by Stampfer et al. (22), except that all cultures were buffered by the addition of 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4. Actinomycin D was added to 5 μg/ml, and [^{3}H]uridine (New England Nuclear Corp.; 5,6-^{3}H, 20 to 40 Ci/mmol) was added to 1 μCi/ml at 30 min postinfection to follow virus-specific RNA accumulation. At 1.5-h intervals, portions of the cultures were transferred into 5% trichloroacetic acid, and the precipitates were collected on membrane filters (Millipore Corp.). The RNA was hydrolyzed from the filters with 0.5 ml of NH_4OH, and the radioactivity was determined by scintillation spectrometry in a dioxane-based scintillation cocktail.

Sucrose gradient analysis of cytoplasmic virus-specific RNA. Infected cells labeled with [^{3}H]uridine were lysed in RSB (10 mM Tris-hydrochloride [pH 7.4]-10 mM NaCl-1.5 mM $MgCl_2$) with 1% Nonidet P-40, and nuclei were removed by centrifugation for 5 min at 1,000 × *g*. The cytoplasmic extract was made 1% with sodium dodecyl sulfate (SDS), layered on a 15 to 30% sucrose gradient in SDS buffer (0.1 M NaCl-10 mM Tris-hydrochloride [pH 7.5]-1 mM EDTA-0.5% SDS), and centrifuged for 11 h at 27,000 rpm at 25°C in a Beckman SW27 rotor. Fractions were collected by pumping from the bottom of the tube through a flow cell in a Gilford spectrophotometer to monitor the absorbance at 260 nm. An equal volume of 25% trichloroacetic acid and 0.1 mg of yeast RNA was added to the samples, and the radioactivity in the precipitate was determined as above.

Pulse-chase labeling of viral proteins. Cells were infected by following the protocol previously described (9), except that infections were buffered by the addition of TES-HEPES (pH 7.4) as described above. At 4 to 5 h postinfection, the cells were harvested, washed with Earle saline solution, and resuspended at 2 × 10^6 cells/ml in Earle saline supplemented with TES-HEPES. The culture was labeled with 6 to 12 μCi of [^{35}S]methionine (New England Nuclear Corp.; 2 to 400 Ci/mmol) per ml for 5 min. Unlabeled methionine was added and the incubation was continued for periods up to 60 min. At specific times, portions of the infected culture were removed and kept at 4°C. The cells were lysed directly in this solution by the addition of 0.1 volume of 10% Nonidet P-40-5% deoxycholate. Nuclei were removed by centrifugation at 1,000 × *g* for 5 min, and the proteins were concentrated from the supernatant by the addition of 9 volumes of acetone. The precipitate was dissolved in gel sample buffer, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described previously (9).

RESULTS

Degradation of specific viral proteins encoded by temperature-sensitive mutants. While the synthesis of viral proteins in cells infected with temperature-sensitive mutant virus strains was being analyzed, it became apparent that certain viral polypeptides accumulated to much lower levels than in cells infected with wild-type virus. To determine whether this deficiency was due to decreased rates of synthesis or increased rates of degradation, we performed a series of pulse-chase experiments (Fig. 1 and 2). Cells infected with wild type or temperature-sensitive strains of virus were pulse-labeled with [^{35}S]methionine for 5 min at either 31°C or after a temperature shift from 31 to 39°C. At this time one-half of the infected culture was removed, and the remainder was incubated with a large excess of unlabeled methionine for 60 min. The viral proteins from the cells and medium were recovered as described above and subjected to polyacrylamide gel electrophoresis. The wild-type viral proteins were stable during the chase period, except that the M protein was partially degraded at 39°C (Fig. 1). At 31°C the wild-type M protein was considerably more stable. The decrease in electrophoretic mobility of the wild-type G protein during the chase period should also be noted. The basis for this change has been shown to involve the addition of sialic acid (9).

The mutant virus *ts*M301(III) demonstrated a lower level of accumulation of the M protein in long labeling periods at 39°C, but when infected cultures were pulse-labeled for 5 min, the M protein was present in approximately normal amounts relative to cells infected with wild-type virus (Fig. 1). After the chase period, the M protein was virtually absent from the culture, indicating a very rapid degradation rate; at 31°C the *ts*M301(III) M protein was much more stable.

Quantitation of the percentage of the original amount of M protein remaining at various times during a 1-h chase period revealed that the *ts*M301(III) M protein had a half-life of 20 min at 39°C, whereas the M protein of wild type and other mutant strains had half-lives of 60 min (Fig. 3). At 31°C the M proteins of all virus strains showed less than 20% turnover during the 60-min chase period.

The M and G proteins of the *ts*M301(III) virus also showed a reduced electrophoretic mobility relative to the wild-type virus proteins [Fig. 1, wild-type and *ts*M301(III) coinfection]. The M and G proteins encoded by *ts*M302(III) and *ts*M303(III) showed the same electrophoretic mobilities as the *ts*M301(III) proteins (D.

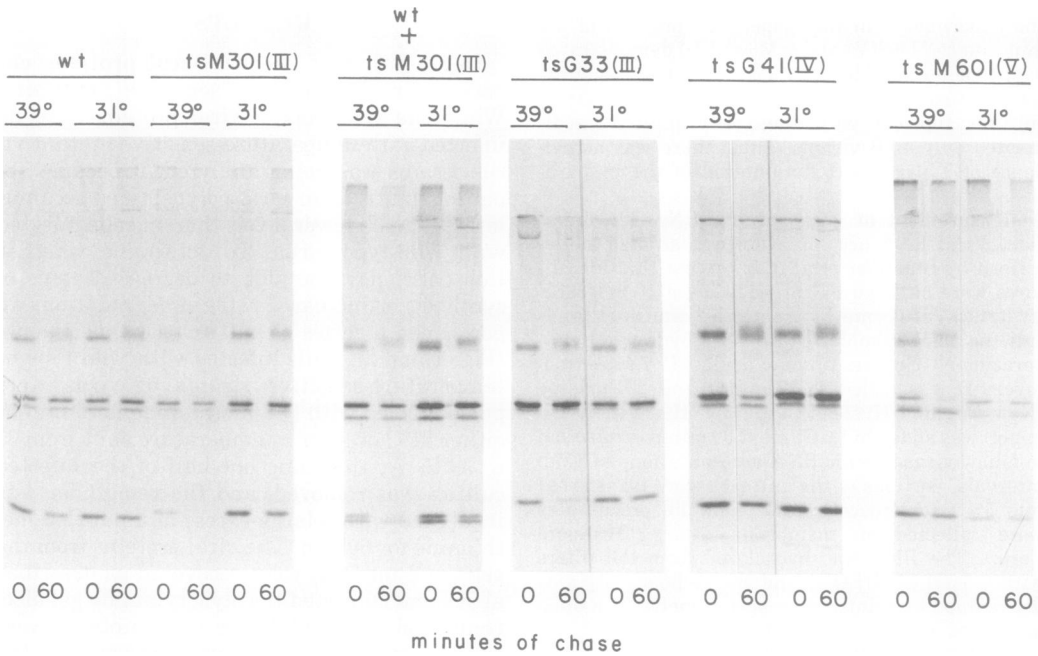

FIG. 1. *Pulse-chase labeling of cultures infected with wild type or temperature-sensitive mutants of VSV. Cultures were infected at a multiplicity of 10 at 31°C. At 4 to 5 h postinfection the cells were collected by centrifugation and resuspended in Earle saline supplemented with TES-HEPES. One-half of each sample was incubated at 39°C and the other half at 31°C. After a 10-min warming period the cultures were labeled for 5 min with [^{35}S]methionine. At that time one-half of each culture was placed at 0 to 4°C, and the remainder was incubated with excess unlabeled methionine for 60 min. The cells were lysed directly in this solution by the addition of 0.1 volume of 10% Nonidet P-40–5% deoxycholate. Nuclei were removed by centrifugation at 1,000 × g for 5 min, and the proteins were concentrated from the supernatant by the addition of 9 volumes of acetone. The precipitate was dissolved in gel sample buffer, and the proteins were analyzed by gel electrophoresis. Exposure times: wt, 24 h; wt + tsM301(III), 36 h; tsG33(III), 39°C, 36 h; 31°C, 24 h; tsG41(IV), 48 h; tsM601(VI), 24 h.*

Knipe, Ph.D. thesis, Massachusetts Institute of Technology Cambridge, 1976), and thus it became necessary to explain how alterations in the mobility of two proteins could be caused by a temperature-sensitive mutation. An identical difference in the electrophoretic mobilities of the proteins of our wild-type virus and tsG33(III) has been found (Knipe, Ph.D. thesis), and we now believe that *ts*M301(III), *ts*M302(III), and *ts*M303(III) all represent accidental reisolates of *ts*G33(III). The *ts*G33(III), like *ts*M301(III), encoded an M protein with an increased degradation rate at 39°C, yet it was more stable at 31°C (Fig. 1). The increased degradation rate of the M protein was not a general property of the Glasgow virus strains because other temperature-sensitive mutants available to us did not show this feature [e.g., *ts*G11(I), *ts*G13(I), and *t*G41(IV); Fig. 1 and 2].

To determine whether the degradation of the M protein in cells infected with *ts*M301(III) was due to the lack of some stabilizing factor or to a defect intrinsic in the protein which made it more susceptible to intracellular proteases, cells were coinfected with wild-type virus and *ts*M301(III) [we will continue to use the term *ts*M301(III), even though we suspect that the virus may actually be *ts*G33(III)]. The *ts*M301(III) M protein, the upper band of the doublet in the M region, was degraded at a faster rate than the wild-type M protein at 39°C, yet at 31°C the two proteins were present at equal levels after the chase period (Fig. 1). Due to the proximity of the two bands (see Fig. 5), it was difficult to determine whether the rate of degradation of the *ts*M301(III) M protein was exactly the same as in singly infected cultures. However, it was obvious that the degradation rate of the *ts*M301(III) M protein was much faster than the M protein of the wild-type virus and that the wild-type virus could not rescue the M protein encoded by the mutant virus. Therefore, we conclude that the M protein of *ts*M301(III), presumably due to a defect in the molecule, is more sensitive to intracellular proteases at the nonpermissive tempera-

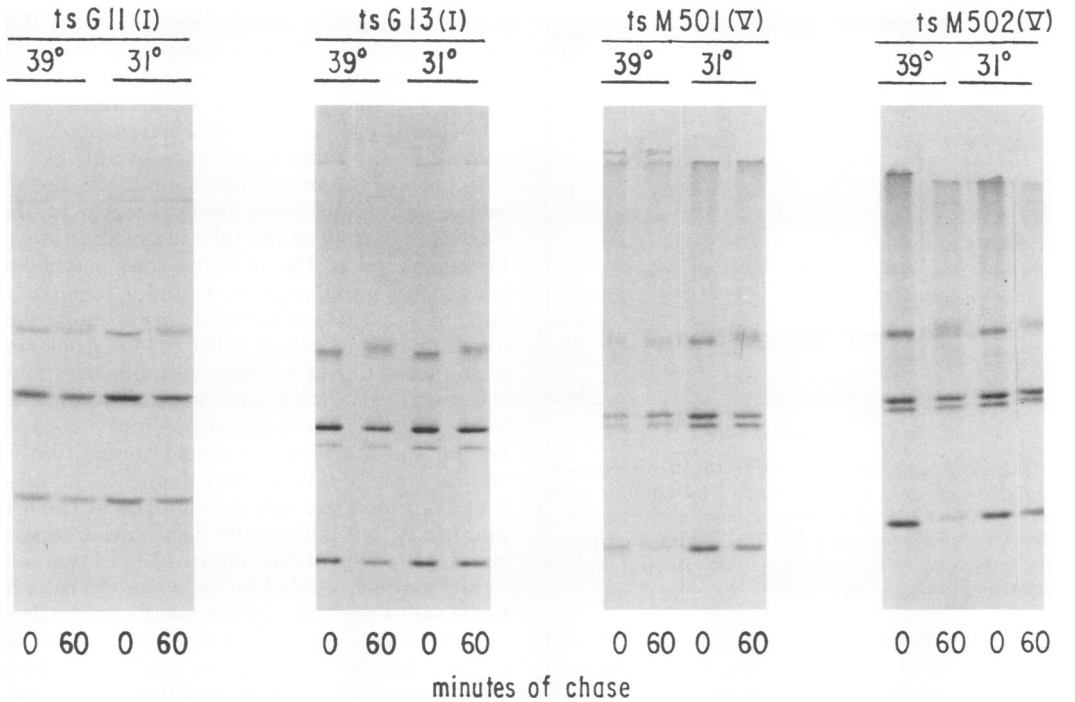

FIG. 2. *Pulse-chase labeling of cultures infected with temperature-sensitive mutants. These experiments were performed as described in the legend to Fig. 1. Exposure times: tsG11(I), 24 h; tsG13(I), 24 h; tsM501(V), 39°C, 72 h; 31°C, 24 h; tsM502(V), 36 h.*

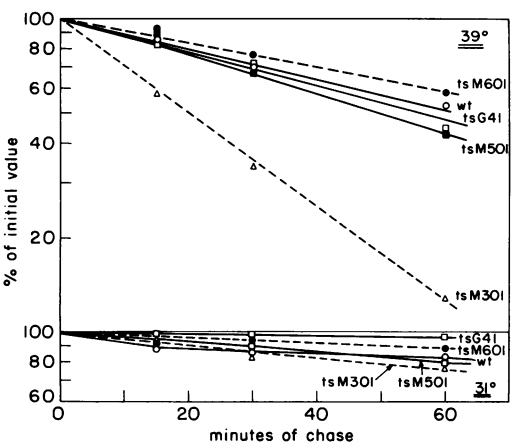

FIG. 3. *Rate of degradation of the M proteins encoded by various virus strains. A pulse-chase experiment similar to the one described in the legend to Fig. 1 was performed, except that portions of the culture were removed at 0, 15, 30, and 60 min of chase. The cytoplasmic and extracellular proteins were concentrated and subjected to polyacrylamide gel electrophoresis. The amount of M protein in each sample was determined and expressed as a percentage of the amount present at the beginning of the chase.*

ture. This conclusion is consistent with the previous suggestion that group III mutations are in the M protein (11).

Mutants were also found that coded for N proteins which were degraded rapidly at the nonpermissive temperature. In cells infected with *ts*G41(IV), the N protein was present in the cells at normal levels after the 5-min pulse-label, but decreased greatly during the chase period at 39°C, indicating a rapid rate of degradation (Fig. 1). However, at 31°C the protein was quite stable. Similarly, cells infected with *ts*M601(VI) showed a nearly complete loss of the N protein during the 60-min chase period at 39°C, but the protein was very stable at 31°C. Determination of the amount of N protein remaining at various periods of chase in cells infected with these mutants revealed that at 39°C the N proteins had half-lives from the initial slope of degradation of approximately 10 min. Cultures infected with wild-type VSV and other mutants showed degradation of less than 20% of the N protein during the 60-min chase period at 39°C. In addition, all of the proteins showed less than 15% degradation at 31°C (Fig. 4). The other mutant that showed this phenotype of a rapidly degrading N protein was *ts*G12(I) (data not shown)—a group I mutant

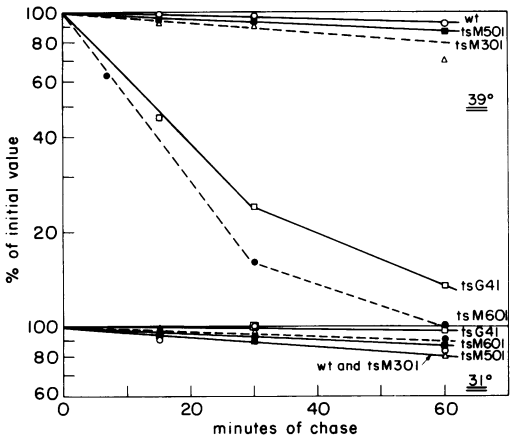

FIG. 4. *Rate of degradation of the N proteins encoded by various virus strains. The amount of N protein in each of the samples from the experiment described in the legend to Fig. 3 was also quantitated and expressed as a percentage of the amount present at the beginning of the chase.*

that nevertheless is defective for replication of 40S viral RNA (16). Therefore, these three mutants shared the property of a thermolabile N protein and a defect in replication of viral RNA, suggesting a link between these two phenotypic properties.

Figure 2 shows examples of another subclass of group I mutants. Pulse-chase experiments with *ts*G11(I) and *ts*G13(I) revealed that the L protein was lost at 39°C, whereas at 31°C the protein was quite stable. This is consistent with the previous assignment of the defect in group I mutants to the L protein (7, 8). The data for the group II mutants are not shown, but we observed no special protein degradation for either *ts*G21(II) or *ts*G22(II). For the group V mutant, *ts*M501, the M protein was degraded at a rate slightly faster than wild type but slower than *ts*M301(III) (Fig. 2 and 3). In addition, a more striking defect was evident: at 39°C the G protein did not undergo the change in mobility ascribed previously to the addition of sialic acid. This will be described in more detail in the accompanying paper (9), but it suggests a defect in the G protein. Mutant *ts*O45(V) showed similar behavior of the G protein (data not shown). An additional property of *ts*M501(V) as well as other group V mutants was that incorporation of [^{35}S]methionine into viral proteins was five- to tenfold higher in cells labeled at 31°C than in cells labeled immediately after a shift from 31 to 39°C (Fig. 2). We are unsure whether this reflects an alteration in protein synthesis or in uptake of the amino acid label.

For the mutant *ts*M502(V), the M protein was degraded at a rate nearly that of *ts*M301(III), and there was no obvious defect in maturation of the G protein. However, analysis of this mutant is further complicated by the fact that it has a defect in RNA synthesis and nucleocapsid assembly (see Table 1 and reference 9).

In each of the experiments described in this section, the cells were lysed directly in the Earle saline used as the labeling medium so as to recover all of the proteins that may have been incorporated into extracellular viral particles. In some sets of samples, presumably due to acetone precipitation of the crude cytoplasm, a small amount of radioactively labeled material remained at the top of the gel. This did not, however, affect the quantitation of the relative amounts of the viral proteins because similar results were obtained from duplicate samples that contained less labeled material at the origin. We do not believe the degradation of specific viral polypeptides described here was due to any artifact of labeling or lysing the cells in Earle saline because similar results on the deficiencies of specific viral proteins were observed in cells labeled in complete medium lacking methionine and washed carefully prior to dissolving the total cells in gel sample buffer (9).

Biochemical analysis of the M and G proteins in cells coinfected with *ts*M301(III) and *ts*M501(V). Because of the mobility difference between the M and G proteins of *ts*M301(III) and *ts*M501(V), we were capable of resolving the gene products of these two mutants by gel electrophoresis even in doubly infected cells. It had been shown previously that the two mutants are capable of complementation, so we examined the fate of the specific gene products in cells coinfected with the two virus strains. At 39°C the M protein of *ts*M301(III) was degraded at a faster rate than the *ts*M501(V) M protein (Fig. 5), whereas at 31°C the two proteins turned over at the same rate. Also, at 39°C the G protein of the *ts*M501(V) virus did not undergo the change of mobility associated with the addition of sialic acid, whereas the G protein of *ts*M301(III) did change in mobility during the 60-min chase period. At 30°C both proteins shifted in mobility. Therefore, it appeared that the defects in *ts*M501(V) and *ts*M301(III) were in the G and M proteins, respectively, and not in *trans*-active secondary proteins. We were able to demonstrate genetic complementation in these cells coinfected with *ts*M301(III) and *ts*M501(V), but we have been unable to detect any increase in viral proteins in total extracellular viral particles (data not shown), presumably due to uninfectious particles produced by the cells. Therefore, we have been unable to

analyze biochemically which viral proteins are assembled into these complemented virions.

RNA synthesis of mutants. To interpret the protein degradation patterns in relation to mutant phenotypes, the synthesis of virus-specific RNA by the various mutants was studied. Table 1 summarizes the accumulation of virus-specific RNA in cells infected with mutant strains and maintained continuously at the nonpermissive and permissive temperatures of 39 and 31°C, respectively. Three mutants were classified as being deficient in RNA synthesis: *ts*M502(V), *ts*M601(VI), and *ts*M602(VI). Mutant *ts*M602(VI) showed RNA synthesis nearly equal to that of wild-type virus at 31°C, but much less at 39°C. On the other hand, *ts*M502(V) and *ts*M601(VI) demonstrated very little RNA synthesis at either 39 or 31°C. This is one possible explanation for their slow growth at 31°C (21).

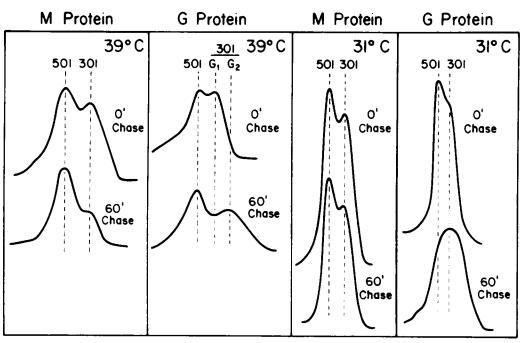

FIG. 5. *Metabolism of the M and G proteins in cells coinfected with tsM301(III) and tsM501(V). A culture was infected with tsM301(III) and tsM501(V), each at a multiplicity of 10 PFU/cell. One-half was incubated at 39°C and the other half at 31°C. At 4 h postinfection both cultures were pulse-labeled with [35S]methionine for 5 min. One-half of each culture was removed and placed at 0 to 4°C. The remainder of each culture was incubated with excess unlabeled methionine for 60 min at the proper temperatures. The total cellular proteins from each sample were analyzed by polyacrylamide gel electrophoresis. The microdensitometer scans from the M and G regions of the gel are shown.*

To identify the nature of the defect in these mutants, we studied the species of virus-specific RNA that synthesized and accumulated in cells infected with these mutants. Infected cultures were labeled for 1 h at 4 h postinfection with [3H]uridine at 31°C or after shifting from 31 to 39°C. Cytoplasmic extracts were prepared as described and sedimented through sucrose gradients. In cells infected with wild-type virus, three species of RNA were synthesized at 39 and 31°C: the 40*S* genome-sized RNA, the 28*S* or L mRNA (13), and the 12–18*S* mRNA's, which code for the G, N, NS, and M proteins (Fig. 6a). Slightly less total synthesis of RNA occurred after a shift to 39°C, especially of the 40*S* and 28*S* species, as previously noted by Perlman and Huang (15).

In cells infected with *ts*M601(VI) and labeled after a shift to 39°C, no 40*S* RNA accumulated, and only a barely detectable amount was observed in cells maintained and labeled at 31°C (Fig. 6b). On the other hand, synthesis of 12–18*S* and 28*S* mRNA was not inhibited to any significant degree by a shift to the nonpermissive temperature. Thus, the lack of replication of 40*S* viral RNA is the probable cause of the low amount of virus-specific RNA synthesis and slow growth, even at 31°C. For the sake of

TABLE 1. *Characteristics of tsM mutants*[a]

Mutant	Virus titer (39°C/31°C ratio)	RNA accumulation at 5 h			RNA synthesis phenotype
		cpm × 10^{-3}		Ratio of 39°C/31°C	
		39°C	31°C		
*ts*M301(III)	3×10^{-5}	19.4	8.6	2.26	+
*ts*M501(V)	3×10^{-6}	7.6	7.0	1.1	+
*ts*M502(V)	10^{-3}	0.0[b]	0.2	—[b]	−
*ts*M601(VI)	6×10^{-3}	0.0[b]	1.4	—[b]	−
*ts*M602(VI)	3×10^{-4}	0.7	9.2	0.08	−
*ts*M5/6-03(V, VI)	6×10^{-4}	4.7	8.2	0.57	+
Wild type	0.4	6.4	11.2	0.57	+

[a] The measurement of virus titer at 39 and 31°C was performed with the first-passage stocks used in all this work. The virus-specific RNA accumulation was measured in infected cultures maintained continuously at 39 and 31°C. [3H]uridine was added at 1 h postinfection, and the acid-precipitable radioactivity was determined in portions of the cultures at 1.5-h intervals thereafter. From the accumulation curve the amount of synthesis of virus-specific RNA was determined, subtracting 600 cpm of actinomycin D-resistant incorporation in mock-infected cells. The RNA synthesis phenotype was designated as negative for any 39°C/31°C ratio of less than 0.1.

[b] No significant detectable incorporation above background at 39°C.

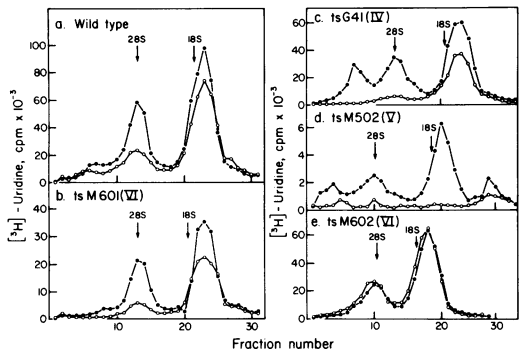

FIG. 6. *Sucrose gradient analysis of RNA species synthesized in cells infected with temperature-sensitive mutants. Cells were infected with the indicated virus strain at a multiplicity of 10 at 31°C and maintained at that temperature until 4 h postinfection. At that time one-half of each culture was transferred to 39°C, and incubation was continued for 10 min. [3H]uridine was then added to 5 μCi/ml in each culture, and the cultures were labeled for 1 h. Cytoplasmic extracts were prepared as described and subjected to centrifugation in 15 to 30% sucrose-SDS gradients. The gradients were collected and the radioactivity was determined in each fraction as described. The positions of sedimentation of the 28S and 18S rRNA's were determined by continuous-flow spectrophotometric monitoring of the absorbance at 250 nm of the gradient. Symbols: ○, RNA synthesized after shift to 39°C; ●, RNA synthesized at 31°C.*

comparison, we have included the RNA synthesis profiles of *ts*G41(IV), a mutant previously described as being deficient in accumulation of 40S viral RNA at the nonpermissive temperature (16). No 40S RNA was found in cells labeled after a shift to 39°C, but a large amount accumulated in cells labeled at 31°C, in contrast to the *ts*M601(VI) mutant (Fig. 6c).

Cells infected with *ts*M502(V) synthesized a lowered amount of all the viral species at 31°C, but in cells labeled after a shift to 39°C very little, if any, of the RNA species was made (Fig. 6d). Thus, it would appear that total RNA synthesis was defective at 39°C. The low amount of RNA synthesized at 31°C was incorporated into the proper species of RNA, and therefore the defect must be some general defect in RNA synthesis, which is apparent even at the permissive temperature.

Analysis of the RNA species synthesized in cells infected with *ts*M602(VI) after a temperature shift to 39°C showed a profile very similar to that from cells labeled at 31°C (Fig. 6e). However, because there was very little 40S RNA apparent in the RNA synthesized at 31°C, we cannot be certain that there was not a defect in the replication of this RNA. It appeared that there was a defect in RNA synthesis only when the entire incubation was at 39°C and not when cultures were incubated for a period of time at 31°C and shifted to 39°C.

DISCUSSION

We have presented evidence in this paper for the rapid degradation of specific viral proteins in cultures infected with certain temperature-sensitive mutants of VSV. In general, the assignment of proteins to complementation groups on the basis of this evidence is consonant with previous assignments on other grounds (see Introduction). We found that the group III mutation lowers the stability of the M protein in a *cis*-active manner; i.e., the increased lability of the protein cannot be corrected by coinfection with our wild-type virus. Mutations in groups I, IV, and VI affect the stability of the N protein. In all likelihood, the N protein is directly affected by mutations in group IV because *ts*M601(VI) did not complement *ts*G41(IV) (21) and may well fall within this group. The complementation behavior of *ts*G12(I) may be anomalous, and its lesion could be in the N protein gene. The L protein in some group I mutants is thermolabile.

Some group V mutations affect the maturation of the G protein. Mutant *ts*M501(V) encodes a G protein that does not undergo the final sialylation at the nonpermissive temperature and cannot be complemented by coinfection with virus strains coding for G proteins that mature normally. Thus, the defect appears to be in the G protein itself.

In previous studies of bacterial protein degradation, protein fragments generated by nonsense mutations or deletions within the structural gene of the protein were shown to have rapid degradation rates (5, 12, 17). Missense mutations have not been clearly documented to cause rapid degradation of mutant proteins in bacteria (4). Eukaryotic cellular temperature-sensitive enzymes have been shown to be degraded at a more rapid rate than the wild-type enzyme (1), but to our knowledge no examples of increased degradation rate of mutant animal virus proteins have been reported. These virus strains could then be useful in the study of protein degradation in cultured animal cells.

We have shown that the degradation of the *ts*M301(III) M protein could not be complemented by coinfection with wild-type virus, and thus the defect appeared to be in the M protein itself. The degradation of the N protein of *ts*G41(IV) or *ts*M601(VI) was not simply the result of lack of viral RNA to assemble the N protein into nucleocapsids because, in cells infected with *ts*M502(V), the N protein remained

soluble and did not assemble into nucleocapsids at 39°C (9), yet it was not rapidly degraded (Fig. 2). In addition, at 31°C in cells infected with *ts*M601(VI) the N protein is quite stable, yet replication of viral RNA is very low (Fig. 6). This suggests that the degradation of the N protein is not a result of the absence of stable 40S RNA. The converse relationship—that the absence of stable 40S RNA is due to defective N protein—seems probable and suggests two possible explanations for the lack of 40S RNA in these cells. The defect in the N protein could prevent the synthesis of 40S RNA at the nonpermissive temperature, or the lack of accumulation of 40S RNA could be due to the inability of the N protein to encapsidate the 40S RNA properly, leaving the RNA susceptible to rapid degradation by intracellular nucleases. We have been unable to find any 40S RNA after short pulse-labels in these cells (data not shown), but it is not possible by these experiments to completely exclude rapid degradation. In vitro systems having the capacity for replication as well as transcription will be required to answer this question.

Our observation that *ts*M601(VI) resembles *ts*G41(IV) in several phenotypic properties raises the question of the present status of complementation group VI. Mutant *ts*M602(VI) appeared to be defective for RNA synthesis when infected cultures were maintained continuously at 39°C, but when cultures were initially infected at 31°C and shifted to 39°C prior to labeling, no defect was apparent in the RNA species synthesized. This conclusion is subject to the reservation that we have been able to detect very little 40S RNA in cells infected with *ts*M602(VI), even at the permissive temperature. The proteins of *ts*M602(VI) show no increased lability, a further difference from *ts*M601(VI). The double mutant *ts*M5/6-03(V, VI) shows no defect in RNA synthesis, but the G protein does not undergo the change in mobility associated with the addition of sialic acid (data not shown). This latter property is probably the result of the group V mutation. Thus, in spite of the apparent similarity between *ts*M601(VI) and *ts*G41(IV), the heterogeneous phenotypes associated with the group VI mutations suggest that group VI may truly be separate from the other complementation groups.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Martin Brock.

D.K. was supported by a National Science Foundation predoctoral fellowship during part of this work and a Public Health Service traineeship during the remainder. H.F.L. was the recipient of Public Health Service research career development award GM-50175 from the National Institute of General Medical Sciences. D.B. is an American Cancer Society research professor. This work was supported by Public Health Service grants AI-08814 and AI-08388 from the National Institute of Allergy and Infectious Diseases, American Cancer Society grant E559, and Public Health Service grant CA-12174 from the National Cancer Institute.

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