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Aberrant Polyadenylation by a Vesicular Stomatitis Virus Mutant Is Due to an Altered L Protein

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***Ts*G16(I) is a temperature-sensitive mutant of vesicular stomatitis virus, Indiana serotype. Our stocks of this mutant overproduce polyadenylic acid in an in vitro transcription system. The overproduction of polyadenylic acid occurs at all temperatures tested (27, 31, 35, and 39°C) and is apparently not due to an alteration in the N protein-RNA template. To characterize the altered moiety in *ts*G16(I) responsible for this phenotype, virions were fractionated and the polyadenylation phenotype in homologous and heterologous reconstitution assays was determined. The aberrant polyadenylation phenotype correlated with the presence of *ts* L protein but not *ts* NS or *ts* M protein fractions. Results of experiments in which solubilized *ts*G16(I) and wild-type virion components were mixed indicated that the altered moiety behaved as if present in stoichiometric amounts relative to active L protein. The effects of raising the temperature from 31 to 39°C in such mixes were as would be predicted upon the assumption that the polyadenylation phenotype was associated with a thermosensitive transcriptase component [the L protein of *ts*G16(I) is known to be thermosensitive]. We conclude that the data strongly support the hypothesis that L is the altered protein responsible for the aberrant polyadenylation phenotype of *ts*G16(I).**

The rhabdovirus, vesicular stomatitis virus (VSV) has a nonsegmented, negative-sense RNA genome with a molecular weight of 3.6×10^6 to 4.0×10^6 (30). Virions contain an RNA-dependent RNA polymerase (transcriptase) which can transcribe the genome in vitro (4), giving rise to five, monocistronic mRNAs (5). VSV codes for five proteins, three of which are required for transcriptive activity: the template is the genomic RNA-N protein complex, and both L and NS proteins also are necessary (14). In addition, the M protein can modulate transcription (9–11, 22, 25). Viral mRNAs made in vitro are capped, methylated (if a suitable methyl donor is present), and polyadenylated (5). These RNA modification reactions are tightly coupled to transcription; it is not known which viral protein(s) is responsible for each modification activity or whether host-coded moieties play a role, although some evidence suggests that virally coded proteins may perform these modifications (2, 5, 6, 12, 15, 16, 23, 24, 28, 29).

In an attempt to elucidate the role of viral proteins in RNA synthesis and modification we have been studying mutants of VSV, Indiana serotype. It has previously been reported (17) that our stock of the temperature-sensitive (*ts*) mutant *ts*G16(I) produces mRNAs in an in vitro transcription system which contain abnormally long tracts of polyadenylic acid [poly(A)]; poly(A) tracts purified from the *ts*G16(I) product RNA migrated on gels as if a high proportion were ca. 5 to 10 times as long as most poly(A) tracts in RNA made by wild-type (*wt*) virions. The 5′ end of *ts*G16(I) mRNAs made in vitro is probably normal since the *ts*G16(I) mRNAs are methylated to almost the same extent as *wt* mRNAs, contain the same methylated cap structures in similar proportions, and are translated as efficiently in a reticulocyte-lysate translation system (17). Since *ts*G16(I) produces such a large excess of poly(A) compared to that produced by *wt* virus in an in vitro transcription system (17), a convenient assay for the aberrant polyadenylation activity is to measure the molar ratio of AMP to UMP incorporated in transcription reactions. *wt* and *ts*G16(I) polyadenylation phenotypes can easily be distinguished with this assay (18; this paper, see Tables 1 to 3).

We have been trying to identify which component of *ts*G16(I) is associated with the aberrant polyadenylation activity. The mutant is known to have a thermolabile transcriptase activity in vitro (20, 27) which is apparently due to a defect in the L protein (19). However, the abnormal polyadenylation does not appear to be temperature dependent (17), and so this phenotype may be due to a second mutation. We have not, as yet, been able to isolate non-*ts* revertants to determine whether the *ts* and aberrant polyadenylation phenotypes are correlated.

When *ts*G16(I) or *wt* virions were separated into pellet (containing N protein-RNA template) and supernatant (containing L, NS, M and G proteins) fractions and then recombined heterologously, the aberrant polyadenylation activity was found to be associated with the supernatant fraction (18). Removal of almost all of the M and G proteins from virions by a gel filtration procedure resulted in the recovery of nucleocapsids which retained L and NS proteins and more than 90% of the transcriptive activity of virions and also still retained the parental polyadenylation phenotype (18). This suggested that aberrant polyadenylation by the mutant was probably associated with the L or NS protein. We, therefore, wished to carry out reconstitution studies in which purified L and NS proteins from mutant or *wt* virions were combined with purified *wt* template to determine whether the aberrant polyadenylation phenotype correlated with the presence of the *ts* L or the *ts* NS fractions.

We report here a modified procedure for preparing purified L and NS proteins which resulted in considerably higher recovery of L protein activity from *ts*G16(I) than previously reported (19) and enabled reconstitution experiments to be done. The results of these and other experiments suggested that an alteration in the L protein accounts for the aberrant polyadenylation phenotype in *ts*G16(I).

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MATERIALS AND METHODS

Virus. Stocks of *wt* VSV (Indiana), originally obtained from the U.S. Department of Agriculture, Beltsville, Md. and *ts*G16 (complementation group I), originally obtained from C. R. Pringle, Warwick, United Kingdom, were kindly supplied by S. U. Emerson and R. R. Wagner, Charlottesville, Va. Virus was grown on BHK-21, clone 13 cells at 31°C as previously described (17). When required, the medium was supplemented with 2.25 μCi of [^{3}H]leucine per ml. Virions were purified by differential, rate zonal, and equilibrium centrifugation (17) and stored in 15% glycerol–10 mM Tris-hydrochloride (pH 7.4) at −18°C. The polyadenylation phenotypes of *wt* and *ts*G16(I) virions, as measured by the ratio of AMP to UMP incorporation (see below), were still maintained after more than a year of storage under these conditions (D. M. Hunt, unpublished data). However, for the experiments reported here the mutant was used within 30 days of harvesting.

Virus was titrated by plaque assay (31) on L cells at 31 and 37°C [which is nonpermissive for *ts*G16(I)]. No *wt* revertants were detected in the stocks of the *ts*G16(I).

Viral protein concentrations were determined by the method of Bradford (8), with bovine plasma gamma globulin as a standard and reagents from Bio-Rad Laboratories, Richmond, Calif.

Preparation of L and NS protein fractions. The method used was based on that of Emerson and Yu (14), but extensive modifications were made to stabilize the L protein of *ts*G16(I) (see below). The final method used was as follows. [^{3}H]leucine-labeled virus (3 to 8 mg) in 15% glycerol–10 mM Tris-hydrochloride (pH 7.4) was treated with an equal volume of 0.5 M NaCl solubilizer (0.5 M NaCl, 0.37% Triton N-101, 20% glycerol, 1.2 mM dithiothreitol [DTT], 4.3 mM Tris-hydrochloride [pH 8.0]) and subjected to gel filtration on a column (1.5 by 19 cm) of agarose A-50m equilibrated with 0.25 M NaCl solubilizer (1 volume of 0.5 M NaCl solubilizer plus 1 volume of 10 mM Tris-hydrochloride [pH 8.0]). The fractions containing transcribing nucleocapsids (TNCs) were pooled, and the NaCl concentration was raised to 0.72 M to dissociate L and NS proteins from the N protein-RNA template. After centrifugation at 160,000 × *g* for 150 min, the supernatant fraction was collected. The NaCl concentration was lowered by gel filtration on a Sephadex G-50 column (1.5 by 19 cm) equilibrated with 0.117 M NaCl column buffer (33% glycerol, 0.07% Triton N-101, 0.2 mM DTT, 33 mM Tris-hydrochloride [pH 7.4], NaCl at the indicated concentration), and the excluded fractions were loaded onto a 10 to 12-ml phosphocellulose column equilibrated with the same buffer. This column was washed with 0.2 M NaCl column buffer, and the L protein was eluted with 1.0 M NaCl column buffer containing 56 μM ATP and 56 μM magnesium acetate. The material which did not bind to phosphocellulose in 0.117 M NaCl column buffer was treated with 3 to 5 ml of phosphocellulose equilibrated with the same buffer and loaded onto a 5 to 8-ml DEAE-cellulose column in the same buffer. The NS fraction was then eluted with 0.4 M NaCl column buffer. L or NS protein-containing fractions were stored as 200-μl samples at −70°C. Storage of L or NS fractions from *wt* or *ts*G16(I) did not result in any detectable change in the polyadenylation phenotype (D. M. Hunt, data not shown). Fractions from the phosphocellulose and DEAE-cellulose columns were collected at a flow rate of ca. 1 and 2 ml per 7 min, respectively, into polypropylene tubes containing 50 μl of a solution containing 5 mg of hemoglobin per ml. All fractions from agarose A-50m, Sephadex G-50, or ion-exchange columns were collected manually at 5°C with the effluent tubing touching the bottom of the collection tube (13).

Preparation of M protein. Virions (ca. 2 mg of protein) were treated with an equal volume of 0.5 M NaCl solubilizer, diluted to 5 ml with 0.25 M NaCl solubilizer, and centrifuged at 160,000 × *g* for 150 min, and the supernatant was collected. The salt concentration was lowered by chromatography on a Sephadex G-50 column equilibrated with 0.117 M NaCl column buffer, and the excluded fractions were loaded on a 10 to 12-ml phosphocellulose column equilibrated with the same buffer. After washing the column with 0.2 M NaCl column buffer, the M protein was eluted with 1.0 M NaCl column buffer containing 56 μM ATP and 56 μM magnesium acetate. The addition of hemoglobin carrier, column flow rates, and collection conditions were as described above.

Template preparation. The template was prepared by a modification of the procedure of Belle Isle and Emerson (7). RNasin (3520 U; Promega Biotec, Madison, Wisc.) was added to 6 to 12 mg of purified *wt* virions in 1 to 3 ml of 15% glycerol–10 mM Tris-hydrochloride (pH 7.4). The virus preparation was treated with an equal volume of 2× high-KCl solubilizer (1.5 M KCl, 20% glycerol, 4% Triton X-100, 1.2 mM DTT, 10 mM Tris-hydrochloride [pH 7.4]), diluted to 176 ml with 1× high-KCl solubilizer (1 volume of 2× high-KCl solubilizer plus 1 volume of 10 mM Tris-hydrochloride [pH 7.4]), incubated at 31°C for 30 min, layered over a 1-ml pad of Renografin-76 and a 2-ml pad of 20% Renografin-76 in 1× high-KCl solubilizer containing 25 U of RNasin per ml, and centrifuged overnight in an SW28 rotor at 78,000 × *g*. The nucleocapsid band was collected from the top of the lower pad, and the Renografin-76 was removed by gel filtration on an agarose A-50m column equilibrated with 1.0 M NaCl–10% glycerol–0.1% Triton X-100–0.1 mM DTT–20 U of RNasin per ml–10 mM Tris-hydrochloride (pH 7.4). The template was then pelleted through a 30% glycerol–1 mM DTT–25 U of RNasin per ml–10 mM Tris-hydrochloride (pH 8.0) layer onto a 100% glycerol pad by centrifugation at 160,000 × *g* for 150 min. The template bands were collected, diluted to 2 ml with 10 mM Tris-hydrochloride (pH 8.0) containing 1 mM DTT and 25 U of RNasin per ml, and stored at −70°C. Stored *wt* L and *wt* NS preparations were used in transcriptase assays to check the purity and activity of each template preparation (if the template is free of L and NS proteins, nucleotide incorporation should require both L and NS proteins to be added). Such determinations also enabled us to use an appropriate dilution of template in reconstitution experiments. The template was usually diluted 5 to 10 times (according to the preparation) with 10 mM Tris-hydrochloride (pH 8.0).

SDS-polyacrylamide gel electrophoresis. Protein containing fractions were prepared for electrophoresis either by precipitation with trichloroacetic acid and washing with acetone (13) or by precipitation with 9 volumes of 4:1 acetone-water at −18°C. The protein precipitates were dissolved in 8 M urea–10 mM Tris-hydrochloride (pH 7.4)–1% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol containing bromophenol blue and heated at 100°C for 5 min before electrophoresis. After electrophoresis by a modification of the method of Laemmli (17, 21), gels were fixed in methanol-water-acetic acid (9:9:2, by volume), rinsed with 50% methanol, dried, sliced, digested at 55°C for 3 h with 9 parts NCS (Amersham Corp., Arlington Heights, Ill.) and 1 part water, and counted by liquid scintillation spectrophotometry after the addition of a xylene-based fluor.

Transcription assays. All transcription assays contained 700 μM ATP, 700 μM CTP, 700 μM GTP, and 60 μM UTP. The required radioisotopes were added at the following activities: [^{3}H]UTP, 10 μCi/ml; [^{3}H]ATP, 100 μCi/ml; [α-^{32}P]UTP, 5 to 10 μCi/ml. Virions were treated with an equal volume of 2× high-NaCl solubilizer (1.44 M NaCl, 20% glycerol, 1.2 mM DTT, 3.74% Triton X-100, 10 mM Tris-hydrochloride [pH 8.0]), 3 volumes of 25% glycerol–10 mM Tris-hydrochloride (pH 8.0), and 5 volumes of 2× nucleotide mix (nucleoside triphosphates at 2× the final concentration, 16 mM magnesium acetate, 1.3 mM DTT, 0.1 M Tris-hydrochloride [pH 8.0]). Reconstitution assays with L and NS fractions contained 3 volumes of diluted template, 2 volumes of NS fraction (or 0.4 M NaCl column buffer containing the same concentration of hemoglobin), 1 volume of L fraction (or 1.0 M NaCl column buffer containing hemoglobin, ATP, and magnesium acetate at the same concentrations as the L fraction; this buffer also was used to dilute the L protein when necessary), and 6 volumes of 2× nucleotide mix.

Incorporation of activity into ice-cold trichloroacetic acid-precipitable material was assayed as previously described (17). This procedure involves elution of the RNA from the filters before the addition of the scintillant. All values are the average of duplicate determinations; unless otherwise stated, all assays were performed at 31°C.

As shown previously (17, 18), a convenient assay for the aberrant polyadenylation phenotype of *ts*G16(I) is to use transcription assays containing [α-^{32}P]UTP, [^{3}H]ATP, and unlabeled CTP and GTP. By comparing the molar amounts of AMP and UMP incorporated into ice-cold trichloroacetic acid-precipitable material the excess amount of AMP relative to UMP incorporated by the mutant compared to that by the *wt* virus is readily distinguished. To determine the picomoles of each radioisotopically labeled compound incorporated, the counts per minute per picomole were determined directly by counting a known volume of reaction mix by liquid scintillation spectrophotometry. We have found that some lots of [α-^{32}P]UTP contain ^{32}P-labeled material which does not behave in transcription assays as if it were in the alpha position of UTP. We, therefore, routinely did a transcription assay in which the incorporation of isotope from [^{3}H]UTP and [α-^{32}P]UTP was compared in a doubly labeled mix. If the picomoles of UMP incorporated were not the same when measured with either isotope, the values obtained with the [^{3}H]UTP were used to normalize the values of counts per minute per picomoles for the [α-^{32}P]UTP.

Chemicals. [5,6-^{3}H]UTP (24 to 40 Ci/mmol), [2,8-^{3}H]ATP (25 to 50 Ci/mmol), [α-^{32}P]UTP (more than 400 Ci/mmol), and [4,5-^{3}H] leucine (30 to 50 Ci/mmol) were from ICN Radioisotopes, Irvine, Calif.; agarose A-50m (coarse) was from Bio-Rad Laboratories; Sephadex G-50-150 (Pharmacia Fine Chemicals, Piscataway, N.J.) was obtained through Sigma Chemical Co., St. Louis, Mo.; P-11 phosphocellulose and DEAE-cellulose (DE 52) were from Whatman Ltd., Maidstone, Kent, United Kingdom; Triton X-100, Triton N-101, and hemoglobin (bovine, type I) were obtained from Sigma Chemical Co.; and Nonidet P-40 (BDH) was obtained from Gallard-Schlesinger, Long Island, N.Y.

RESULTS

Purification of L protein. When *ts*G16(I) L protein is purified by the method of Hunt et al., it retains little or no transcriptive activity (19). We therefore sought for ways to stabilize this activity.

We have used TNCs prepared by agarose A-50 m gel filtration as the material for enzyme solubilization, as these can be prepared rapidly and contain little or no M or G protein (18). The method for purification of TNCs previously described (18) was modified by the replacement of Triton X-100 (1.87%) by Triton N-101 (0.187%) in the column buffer, since we found that the transcriptive activity of *ts*G16(I) TNCs was very unstable when TNCs were stored in buffers containing ≥0.6% Triton X-100 but far more stable when stored in Triton N-101–containing buffers. (It has been reported [26, 27] that Triton X-100 can affect the heat stability of *wt* transcriptive activity). Other modifications made in an attempt to increase the yield of transcriptionally active *ts* L protein were to use Sephadex G-50 filtration rather than dialysis to lower the NaCl concentration before ion-exchange chromatography, to use Triton N-101 rather that Triton X-100 in the column buffers for ion-exchange chromatography, and to add 56 μM ATP and 56 μM magnesium acetate to the 1.0 M NaCl column buffer used to elute the L protein.

*Ts*G16(I) L- and NS-containing fractions retained considerable transcriptive activity when purified by this method, either when assayed immediately upon purification (Table 1) or when assayed after storage at −70°C (Hunt, unpublished data).

Results of reconstitution assays with L and NS fractions. Since it has already been shown (18) that the aberrant polyadenylation phenotype of *ts*G16(I) is due to a component of the enzyme fraction rather than the template fraction, all reconstitutions were done with a *wt* template fraction. *wt* L, *wt* NS, *ts* L, or *ts* NS fractions were always

TABLE 1. In vitro transcriptive activity and polyadenylation phenotype of unfractionated *wt* or *ts*G16(I) virions, and of reconstitution assays with L and NS fractions derived from these virions, when assayed in various combinations[a]

Virion components[b]	Picomoles incorporated[c]		Ratio (AMP/UMP)
	AMP	UMP	
Unfractionated *wt*	1,770.1	1,361.5	1.30
Unfractionated *ts*	1,883.5	803.1	2.35
wt T + *wt* L-1 + *wt* NS	154.9	109.3	1.42
wt T + *ts* L + *ts* NS	84.5	27.2	3.11
wt T + *wt* L-1 + *ts* NS	147.2	105.6	1.39
wt T + *ts* L + *wt* NS	59.4	21.0	2.83
wt T + *wt* L-2 + *wt* NS	258.6	188.2	1.37
wt T + *wt* L-2 + *ts* NS	285.6	208.1	1.37

[a] Transcription reactions containing CTP, GTP, [^{3}H]ATP, and [α-^{32}P]UTP were incubated at 31°C for 120 min, and the incorporation of each isotope into ice-cold trichloroacetic acid-precipitable material was determined by dual-channel liquid scintillation spectrophotometry.

[b] Unfractionated virus was treated with an equal volume of 2× high-NaCl solubilizer and assayed as described in the text. All reconstitutions of subviral fractions contained the same concentration of purified *wt* template (*wt* T). *wt* L-2 is an undiluted *wt* L preparation and was used to determine whether the amount of L fraction present was limiting in assays with *wt* L-1 or *ts* L.

[c] No correction has been made for each L or NS fraction assayed separately in the presence of *wt* T. When assayed in the presence of *wt* T: *wt* NS, *ts* L, or *ts* NS fractions incorporated less than 0.9 pmol of UMP in 120 min, *wt* L-1 incorporated 7.2 pmol, and *wt* L-2 incorporated 6.9 pmol. The percentages of the total UMP incorporation in assays containing both L and NS fractions which was observed with the fractions assayed separately with *wt* T were 7.2% (*wt* L-1 plus *wt* NS), 0.9% (*ts* L plus *ts* NS), 6.8% (*wt* L-1 plus *ts* NS), 1.1% (*ts* L plus *wt* NS), 4.0% (*wt* L-2 plus *wt* NS), and 3.3% (*wt* L-2 plus *ts* NS).

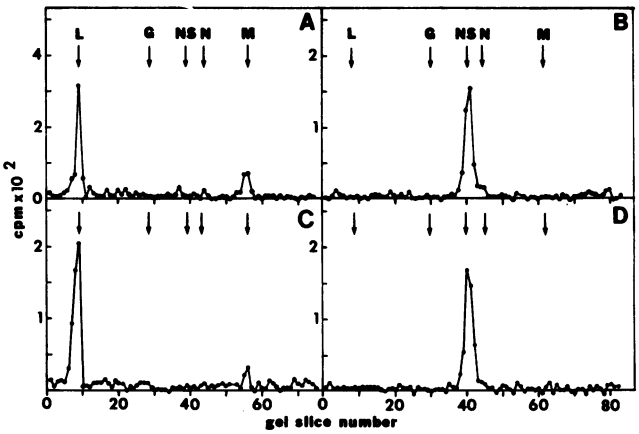

FIG. 1. SDS-polyacrylamide gel electrophoresis of [^{3}H]leucine-labeled protein present in L or NS fractions derived from *wt* or *ts*G16(I) virions. L (A and C) or NS (B and D) fractions were prepared from [^{3}H]leucine-labeled *wt* (A and B) or *ts*G16(I) (C and D) virions and subjected to SDS-polyacrylamide gel electrophoresis. Virion marker proteins were run in parallel lanes. The fractions shown are those used in the reconstitutions shown in Table 1 (the *wt* L gel [A] corresponds to *wt* L-1).

tested for purity by polyacrylamide gel electrophoresis and by assaying each separately with *wt* template (no incorporation should be observed since RNA synthesis requires template plus L plus NS proteins [14]). Background incorporation attributable to impure fractions was no more than 7.2% of the incorporation seen when L, NS, and template fractions were all present (see the legend to Table 1).

When L and NS fractions were recombined in an homologous fashion, the polyadenylation phenotype (as measured by the ratio of picomoles of AMP to picomoles of UMP incorporated) of the parental virus was maintained (Table 1). The increase in the ratio of AMP to UMP seen in the homologous *ts*G16(I) reconstitutions compared with unfractionated virions has been repeatedly observed. A lesser increase in the case of *wt* homologous reconstitutions compared with *wt* virions was sometimes but not always seen.

When L and NS fractions (Fig. 1) were combined in an heterologous fashion the combination (*wt* template plus *wt* L plus *ts* NS) had a *wt* polyadenylation phenotype, but the combination (*wt* template plus *ts* L plus *wt* NS) had a mutant polyadenylation phenotype (Table 1), suggesting that the aberrant polyadenylation phenotype of *ts*G16(I) is due to a component which is, or which copurifies with, the L protein.

Effect of L protein concentration in reconstitution assays. Since *ts* L protein fractions were the limiting factor in those reconstitutions in which they were present, the *wt* L fraction used in the main part of the reconstitution experiment shown in Table 1 (*wt* L-1) was diluted such that it was rate limiting in those assays in which it was present. To show that the NS fractions and template used in the heterologous reconstitutions (Table 1) could indeed support more incorporation than that observed when they were combined with *wt* L-1 or *ts* L, they were also assayed with a more active preparation of *wt* L(*wt* L-2, bottom two lines of Table 1), and an almost twofold increase in UMP incorporation was observed. Furthermore, lowering the concentration of *wt* L in reconstitution assays such that the UMP incorporation activity was similar to that of the *ts* L-containing reconstitutions in Table 1 still resulted in a *wt* polyadenylation phenotype (data not shown).

Does M protein affect the polyadenylation phenotype? One interpretation of the above data is that the aberrant polyadenylation phenotype of *ts*G16(I) is due to an alteration in the L protein, but other explanations are possible. The L protein fractions but not the NS protein fractions contained low amounts of M protein (Fig. 1). To exclude the possibility that M was the altered protein, M was purified from *wt* or *ts*G16(I) virions and added to homologous or heterologous TNCs such that the ratio of M protein to N protein was approximately the same as that in virions. Although M protein is present in very low amounts in TNCs (Fig. 2A and C), some M may be present since L fractions prepared from such TNCs often contain detectable amounts of M protein (Fig. 1A and C); we therefore added purified M protein fractions (Fig. 2B and D) to TNCs in the presence of 0.725 M NaCl so that any M bound to the TNCs would be dissociated from the template, and thus the large excess of added M protein would be able to compete for binding sites once the NaCl concentration was lowered before assay of nucleotide incorporation. The results indicated that the ratio of AMP to UMP incorporated by TNCs was similar to that of parental virions whether no M protein was added, *wt* M protein was added, or *ts* M protein was added (data not shown). The small amount of *ts* L protein present in the *ts* M fractions (Fig. 2D) was apparently insufficient to alter the ratio of AMP to UMP in these experiments.

Could a host factor be involved in the aberrant polyadenylation phenotype? Another possible explanation for the reconstitution data (Table 1) could be that there is a change in the spectrum of host components packaged by the mutant and that there is a host factor involved in the regulation of poly(A) synthesis which copurifies with L protein. The aberrant polyadenylation phenotype of *ts*G16(I) could then be due to the presence of an additional host factor in the *ts*

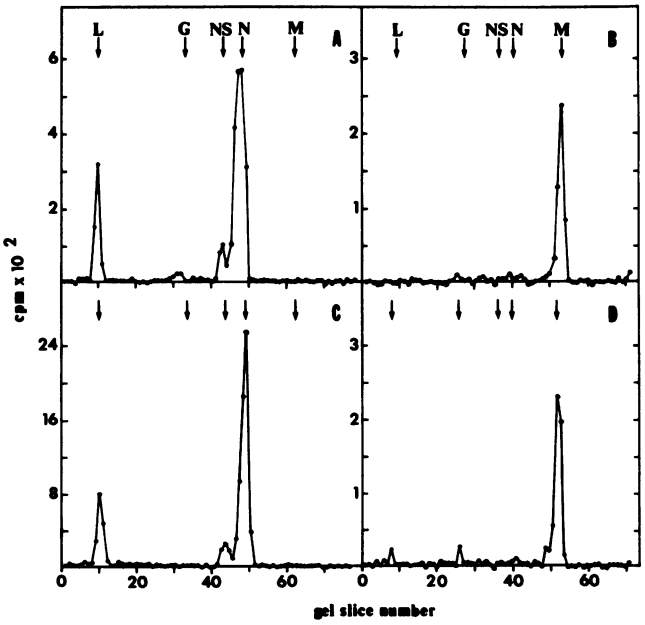

FIG. 2. SDS-polyacrylamide gel electrophoresis of [^{3}H]leucine-labeled protein present in TNC or M fractions derived from *wt* or *ts*G16(I) virions. TNC (A and C) or M (B and D) fractions were prepared from [^{3}H]leucine-labeled *wt* (A and B) or *ts*G16(I) (C and D) virions and then subjected to SDS-polyacrylamide gel electrophoresis. Virion marker proteins were run in parallel lanes.

TABLE 2. Effect of mixing *wt* and *ts*G16(I) solubilized virion components in various proportions upon incorporation of AMP and UMP in vitro

Virus (μg of protein per ml in final mix)[a]	Picomoles incorporated[b]		Ratio (AMP/UMP)
	AMP	UMP	
wt (150)	626.9	408.2	1.54
ts (150)	719.8	270.3	2.66
Predicted values[c] from above for:			
wt (120) + *ts* (30)	645.5	380.7	1.70
wt (90) + *ts* (60)	664.0	353.0	1.88
wt (60) + *ts* (90)	682.7	325.5	2.10
wt (30) + *ts* (120)	701.2	297.8	2.35
Actual values obtained for:			
wt (120) + ts (30)	620.3	372.2	1.67
wt (90) + ts (60)	723.9	394.4	1.84
wt (60) + ts (90)	655.3	318.7	2.06
wt (30) + ts (120)	674.3	292.3	2.31

[a] Virions were mixed in the indicated proportions (at 10 times the final protein concentration), then treated with an equal volume of 2× high-NaCl solubilizer, diluted, and assayed in mixes containing GTP, CTP, [^{3}H]ATP, and [α-^{32}P]UTP. The final protein concentration in all assays was 150 μg/ml.

[b] All incubations were done at 31°C for 120 min. Incorporation of [^{3}H]AMP and [α-^{32}P]UMP was determined by dual-channel liquid scintillation spectrophotometry.

[c] Predicted values were determined for AMP or UMP incorporation by assuming that if the concentration of a virus in a mix was *x* micrograms per milliliter then the incorporation of each isotope would be (*x*/150) × (incorporation of that isotope by that virus assayed separately at 150 μg of protein per ml). The expected contribution for *wt* and *ts* virus was then added to give the values in the Table, and the expected ratio of AMP to UMP was calculated by using these figures.

virions (and *ts* L fractions) or to the loss of a host factor normally packaged by *wt* virions. If this were so, one might expect that if solubilized *ts* and *wt* virions were mixed in the presence of 0.72 M NaCl (to facilitate exchange of factors), then the putative host factor would influence both *wt* and *ts* polymerases, and all the RNA made would exhibit the *wt* polyadenylation, or the aberrant polyadenylation, pattern (according to whether the putative host factor is thought to be present in *wt* or *ts* virions). However, if aberrant polyadenylation by *ts*G16(I) is a property of the L protein itself, then upon mixing the two viruses one might expect that the product RNA would have an ratio of AMP to UMP which could be predicted by assaying the incorporation of these nucleotides by each virus alone and using these values to assess the contribution expected to be made by each in the mixture; the results of such a mixing experiment (Table 2) showed close agreement between the predicted ratio of AMP to UMP incorporation and that determined experimentally.

If the aberrant polyadenylation in *ts*G16(I) is due to an alteration in the L protein then, since it is known that the L protein in *ts*G16(I) is thermosensitive (19), it might be expected that if solubilized *wt* and *ts* virions were mixed in the presence of 0.72 M NaCl (to allow exchange of components) that, upon raising the temperature to 39°C, the contribution of the *ts* components to both UMP and AMP incorporation would be decreased and would be equivalent to that observed if *ts*G16(I) were assayed in the absence of *wt* components. If, however, *ts*G16(I) contains a host component which causes aberrant polyadenylation, this component would not be expected to show the same thermosensitivity as *ts*G16(I) and should be able to exert its influence to some degree on the RNA made at 39°C by the transcription complexes containing *wt* L protein. The results of an experiment in which *wt* virus, *ts* virus, or (*wt* plus *ts*) virus was assayed for the ratio of AMP to UMP incorporation at 31 and 39°C are shown in Table 3. The incorporation of AMP relative to UMP seen was in close agreement with that predicted on the assumption that the aberrant polyadenylation was due to an alteration in a protein which was also temperature sensitive.

DISCUSSION

*Ts*G16(I) is a temperature-sensitive mutant of VSV, Indiana serotype, which has a thermosensitive L protein in in vitro transcription assays (19). Our stocks of this mutant also exhibit an aberrant polyadenylation phenotype under in vitro transcription conditions in that the RNA made contains a high proportion of AMP residues in poly(A). There is no evidence that the aberrant polyadenylation phenotype of *ts*G16(I) is temperature dependent, it being expressed at all temperatures tested (17; this paper, Table 3). We do not know whether the temperature-sensitive and polyadenylation phenotypes of *ts*G16(I) are due to the same mutation, as non-*ts* revertants of *ts*G16(I) have not yet been isolated.

To identify the altered component in *ts*G16(I) responsible for the aberrant polyadenylation phenotype, we have done experiments involving protein fractionation and reconstitution of subviral fractions. Previous studies (17, 18) have shown that the moiety in *ts*G16(I) associated with the polyadenylation phenotype was apparently not a component of the N protein-RNA template and was unlikely to be either the M or G protein since transcribing nucleocapsid preparations, from which virtually all of the M and G proteins had been removed, retained the mutant polyadenylation pheno-

TABLE 3. Effect of 31 versus 39°C incubation on the ratio of AMP to UMP incorporation by *wt* virus, *ts*G16(I) virus, or [*wt* plus *ts*G16(I)] virus

Virus (protein concn)[a]	Incubation		Picomoles incorporated[b]		Ratio (AMP/UMP)
	°C	min	AMP	UMP	
wt (150 μg/ml)	31	30	108.4	70.8	1.53
	31	60	267.8	178.2	1.50
	39	30	79.3	56.8	1.40
	39	60	124.1	91.6	1.35
*ts*G16(I) (150 μg/ml)	31	30	95.2	41.7	2.28
	31	60	190.8	78.1	2.44
	39	30	24.1	9.8	2.46
	39	60	27.7	11.5	2.41
Predicted values[c] from above for [*wt* + *ts*G16(I)], both at 75 μg/ml	31	30	101.8	56.3	1.81
	31	60	229.3	128.2	1.79
	39	30	51.7	33.3	1.55
	39	60	75.9	51.6	1.47
Actual values obtained for [*wt* + *ts*G16(I)], both at 75 μg/ml	31	30	106.5	61.3	1.74
	31	60	248.1	138.8	1.79
	39	30	57.3	37.9	1.51
	39	60	97.5	66.7	1.46

[a] Virions were treated with 2× high-NaCl solubilizer and then diluted and assayed in mixes containing radiolabel in both ATP and UTP. The final protein concentration in the assay mix was 150 μg/ml in all cases.

[b] Incorporation of [^{3}H]AMP and [α-^{32}P]UMP was determined by dual-channel liquid scintillation spectrophotometry.

[c] Predicted values were determined for AMP or UMP incorporation by assuming that the contribution made by each virus in the mix would be equal to 50% of that determined under the same incubation conditions for each virus assayed separately at 150 μg of protein per ml.

type. These data suggested that either the L or the NS protein was most likely to be responsible for the mutant polyadenylation phenotype.

Purification of active L protein from *ts*G16(I) was problematical, but various modifications to the previously published procedures (14, 19) enabled us to prepare L protein fractions from the mutant which were sufficiently active to use in reconstitution experiments. The polyadenylation phenotype was assayed by measuring the ratio of picomoles of AMP incorporated to picomoles of UMP incorporated in transcription assays in which both ATP and UTP were radiolabeled. By using such an assay the excess AMP incorporation by *ts*G16(I) compared to that by *wt* is readily detected. The results of reconstitution experiments with purified L- and NS-protein-containing fractions showed that the aberrantly high incorporation of AMP relative to UMP correlated with the presence of *ts* L fractions but not *ts* NS fractions (Table 1).

Although the altered factor involved in aberrant polyadenylation copurified with the L protein, it might not be the L protein itself. Both *wt* and *ts* L fractions contained low concentrations of M protein (Fig. 1A and C), and it was possible that *ts*G16(I) M protein could modulate poly(A) synthesis, even when present at a low concentration. However, experiments in which purified M protein fractions were added to transcribing nucleocapsids suggested that this was not so.

Another possibility is that the mutation in *ts*G16(I) associated with the aberrant polyadenylation phenotype results in a change in the spectrum of host components packaged in the virion and that there is a host component involved in the regulation of poly(A) synthesis which copurifies with L protein under our conditions. Thus, the polyadenylation phenotype of *ts*G16(I) could be due to the presence of an additional host factor in *ts* L fractions or to the loss of a factor normally present in *wt* L fractions. Thus, if *wt* and *ts* virions were mixed under conditions in which transcriptase components are dissociated from the template (to facilitate exchange of factors) and then assayed for incorporation of AMP relative to UMP, one might expect that the ratio would be that typical of *wt* virus (if the factor is normally present in *wt* virions) or that typical of the *ts* virus (if the factor is only present in *ts* virions). However, if the polyadenylation phenotype is due to the *ts* L protein itself, the product RNA in the mixed assay might be expected to have an intermediate phenotype which could be predicted by assaying the activity of *ts* and *wt* virions separately. The observed results (Table 2) were consistent with those predicted, using the hypothesis that the *ts* L protein was the protein responsible for the aberrant AMP incorporation. If this hypothesis were correct, one would also predict that if *wt* and *ts* virions were mixed and assayed at 31 or 39°C the ratio of AMP to UMP incorporated should decrease at the higher temperature since the *ts* L protein is more thermosensitive than the *wt* L protein and will make a lesser contribution to RNA synthesis at 39°C relative to 31°C. However, if *ts*G16(I) contained a host factor which modulated poly(A) synthesis, the factor would not be expected to be as thermosensitive as the *ts* L protein and would be expected to affect polyadenylation at 39°C, even though the RNA would be predominantly made under the influence of the *wt* L protein (this assumes that the putative host factor is not so tightly associated with the *ts* L protein that it can only function in a *cis* fashion). Again, the experimental results (Table 3) were consistent with those predicted by using the hypothesis that the altered protein in *ts*G16(I) is the L protein.

It is extremely difficult (if not impossible) to completely exclude the possibility of host factor involvement in the difference in polyadenylation phenotype between *wt* and *ts*G16(I) virions under in vitro transcription conditions, but, in view of the above data, all the results appear to be fully consistent with the hypothesis that an altered L protein in *ts*G16(I) is responsible for the aberrant polyadenylation phenotype, and we conclude that this is the most probable explanation of the data.

We would like to emphasize that the above results do not show that the L protein is necessarily the poly(A) polymerase. The data only indicate that mutations in the L protein can affect polyadenylation. This could be a direct effect because the L protein is, or directly modulates the activity of, the poly(A) polymerase, or it could be an indirect effect. For example, since transcription by VSV is sequential (1, 3), mutations which affect initiation events at each gene might result in increased polyadenylation of the preceding gene; we are currently investigating this possibility.

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LITERATURE CITED

1. **Abraham, G., and A. K. Banerjee.** 1976. Sequential transcription of the genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73**:1504–1508.
2. **Abraham, G., and A. K. Banerjee.** 1976. The nature of the RNA products synthesized *in vitro* by subviral components of vesicular stomatitis virus. Virology **71**:230–241.
3. **Ball, L. A., and C. N. White.** 1976. Order of transcription of genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73**:442–446.
4. **Baltimore, D., A. S. Huang, and M. Stampfer.** 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci. U.S.A. **66**:572–576.
5. **Banerjee, A. K., G. Abraham, and R. J. Colonno.** 1977. Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. **34**:1–8.
6. **Banerjee, A. K., S. A. Moyer, and D. P. Rhodes.** 1974. Studies on the *in vitro* adenylation of RNA by vesicular stomatitis virus. Virology **61**:547–558.
7. **Belle Isle, H. D., and S. U. Emerson.** 1982. Use of a hybrid infectivity assay to analyze primary transcription of temperature-sensitive mutants of the New Jersey serotype of vesicular stomatitis virus. J. Virol. **43**:37–40.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
9. **Carroll, A. R., and R. R. Wagner.** 1979. Role of the membrane (M) protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus. J. Virol. **29**:134–142.
10. **Clinton, G. M., S. P. Little, F. S. Hagen, and A. S. Huang.** 1978. The matrix (M) protein of vesicular stomatitis virus regulates transcription. Cell **15**:1455–1462.
11. **De, B. P., G. B. Thornton, D. Luk, and A. K. Banerjee.** 1982. Purified matrix protein of vesicular stomatitis virus blocks viral transcription *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **79**:7137–7141.
12. **Deutsch, V., and A. K. Banerjee.** 1979. Effect of temperature on the enzymatic activities present in purified virions of vesicular stomatitis virus. Biochem. Biophys. Res. Commun. **88**:1360–1367.
13. **Emerson, S. U., and R. R. Wagner.** 1973. L protein requirement for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **12**:1325–1335.

14. **Emerson, S. U., and Y.-H. Yu.** 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **15**:1348–1356.
15. **Herman, R. C., M. Schubert, J. D. Keene, and R. A. Lazzarini.** 1980. Polycistronic vesicular stomatitis virus RNA transcripts. Proc. Natl. Acad. Sci. U.S.A. **77**:4662–4665.
16. **Horikami, S. M., and S. A. Moyer.** 1982. Host range mutants of vesicular stomatitis virus defective in *in vitro* RNA methylation. Proc. Natl. Acad. Sci. U.S.A. **79**:7694–7698.
17. **Hunt, D. M.** 1983. Vesicular stomatitis virus mutant with altered polyadenylic acid polymerase activity in vitro. J. Virol. **46**:788–799.
18. **Hunt, D. M.** 1984. Characterization of a mutant of vesicular stomatitis virus with an aberrant *in vitro* polyadenylation activity, p. 123–130. *In* D. H. L. Bishop and R. W. Compans (ed.), Nonsegmented negative strand viruses. Academic Press, Inc., Orlando, Fla.
19. **Hunt, D. M., S. U. Emerson, and R. R. Wagner.** 1976. RNA^- temperature sensitive mutants of vesicular stomatitis virus: L-protein thermosensitivity accounts for transcriptase restriction of group I mutants. J. Virol. **18**:596–603.
20. **Hunt, D. M., and R. R. Wagner.** 1974. Location of the transcription defect in group I temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. **13**:28–35.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Martinet, C., A. Combard, C. Printz-Ané and P. Printz.** 1979. Envelope proteins and replication of vesicular stomatitis virus: in vivo effects of RNA^+ temperature-sensitive mutations on viral RNA synthesis. J. Virol. **29**:123–133.
23. **Murphy, M. F., and R. A. Lazzarini.** 1974. Synthesis of viral mRNA and polyadenylate by a ribonucleoprotein complex from extracts of VSV-infected cells. Cell **3**:77–84.
24. **Naito, S., and A. Ishihama.** 1976. Function and structure of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. **251**:4307–4314.
25. **Pinney, D. F., and S. U. Emerson.** 1982. In vitro synthesis of triphosphate-initiated N-gene mRNA oligonucleotides is regulated by the matrix protein of vesicular stomatitis virus. J. Virol. **42**:897–904.
26. **Piwnica-Worms, H., and J. D. Keene.** 1983. Sequential synthesis of small capped RNA transcripts *in vitro* by vesicular stomatitis virus. Virology **125**:206–218.
27. **Szilágyi, J. F., and C. R. Pringle.** 1972. Effect of temperature-sensitive mutations on the virion-associated RNA transcriptase of vesicular stomatitis virus. J. Mol. Biol. **71**:281–291.
28. **Testa, D., and A. K. Banerjee.** 1977. Two methyltransferase activities in the purified virions of vesicular stomatitis virus. J. Virol. **24**:786–793.
29. **Villarreal, L. P., and J. J. Holland.** 1973. Synthesis of poly(A) *in vitro* by purified virions of vesicular stomatitis virus. Nat. New Biol. **246**:17–19.
30. **Wagner, R. R.** 1975. Reproduction of rhabdoviruses, p. 1–93. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 4. Plenum Publishing Corp., New York.
31. **Wagner, R. R., A. H. Levy, R. M. Snyder, G. A. Ratcliff, Jr., and D. F. Hyatt.** 1963. Biologic properties of two plaque variants of vesicular stomatitis virus (Indiana serotype). J. Immunol. **91**:112–122.