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Vesicular Stomatitis Virus Mutant with Altered Polyadenylic Acid Polymerase Activity In Vitro

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In vitro RNA synthesis by purified virions of a stock of *ts*G16(I) was aberrant compared with that of wild-type (*wt*) vesicular stomatitis virus. RNA made in vitro by *ts*G16(I) contained a larger proportion of A residues in polyadenylic acid [poly(A)] tracts than did RNA synthesized by *wt* virus, *ts*G13(I), *ts*G21(II) or *ts*G41(IV). Experiments to determine whether the aberrant polyadenylation was correlated with the known thermolability of the *ts*G16(I) L protein were inconclusive. Total product RNA made by *ts*G16(I) was methylated to almost the same extent as *wt* RNA, contained the same major methylated 5′ cap structure as *wt* RNA, and was translated as well in a reticulocyte cell-free system, yielding the same molecular weight proteins in similar ratios. Most polyadenylated [poly(A)$^+$] RNA made by *ts*G16(I) was considerably larger than *wt* poly(A)$^+$ RNA and richer in AMP:UMP residues; however, the protein-coding capacities of mutant and *wt* poly(A)$^+$ RNAs were similar. This suggested that most mRNAs made in vitro by *ts*G16(I) might possess very long poly(A)$^+$ tracts, and digestion of RNA by T_1 RNase supported this. It appeared, therefore, that a virally coded component of vesicular stomatitis virus could affect polyadenylation. This could be the poly(A) polymerase itself, a protein involved in control of polyadenylation, or a protein which affects an event spatially and temporally connected with polyadenylation (such as initiation of the subsequent mRNA).

The genome of the rhabdovirus vesicular stomatitis virus (VSV) is a nonsegmented, negative-sense RNA of molecular weight 3.6×10^6 to 4.0×10^6 (32). Virions contain five virally coded proteins, including an RNA-dependent RNA polymerase (transcriptase) (4) which transcribes the genome in vitro, giving rise to five monocistronic mRNAs (5, 8). Transcription requires three viral proteins. The template is genomic RNA complexed tightly with N protein; L and NS proteins are both required for transcriptase activity (14, 15). Viral mRNAs made in vitro are capped and methylated, having the 5′ terminal sequence m^{7}G(5′)ppp(5′)Amp, and are polyadenylated [poly(A)$^+$]. These modification reactions are tightly coupled to transcription, but it is not known whether they are carried out by host- or virally coded enzymes, although any proteins necessary must be present in purified virions, and the evidence tends to suggest that they may be virally coded (1, 2, 5–8, 12, 17, 24, 29, 31).

In an attempt to elucidate the processes of viral transcription and to determine which, if any, viral proteins are involved in RNA modification, I am surveying various aspects of in vitro RNA synthesis by temperature-sensitive (*ts*) mutants of VSV, Indiana serotype, belonging to complementation groups I, II, and IV, which are thought to represent mutants with alterations in the three proteins required for transcription, i.e., L, NS, and N, respectively (25).

*ts*G16(I) is a *ts* mutant belonging to complementation group I. The temperature-sensitive defect in this mutant has been shown to be correlated with a thermolabile L protein (18). I present evidence here that polyadenylation of mRNA by our stocks of *ts*G16(I) in in vitro transcription reactions is aberrant.

MATERIALS AND METHODS

Virus. The Glasgow strain of wild-type (*wt*) VSV (Indiana) and *ts* mutants isolated in Glasgow, *ts*G16(I), *ts*G13(I), *ts*G21(II), and *ts*G41(IV), were kindly supplied by R. R. Wagner, Charlottesville, Va., who received them from C. R. Pringle, Glasgow, Scotland. The roman numerals indicate the complementation group to which the mutants belong. Recently cloned stocks of *wt* virus and *ts* mutant virus were grown at 31°C on confluent monolayers of BHK-21 clone 13 cells in 75-cm^2 plastic flasks in the presence of Dulbecco modified Eagle medium (4,500 mg of glucose/liter). Cultures were infected at a multiplicity of 1 PFU per cell, and virions were harvested 18 to 22 h postinfection. Purified virions were prepared by differential, rate zonal, and equilibrium centrifugation as described previously (20), with the exception that the gradients

used in the equilibrium centrifugation were 0 to 32% potassium tartrate and 20 to 0% glycerol and contained 1 M NaCl–10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA. Virions were stored in 15% glycerol–10 mM Tris-hydrochloride (pH 7.4) at −18°C until needed; *ts* mutants were used within 30 h of harvesting.

Virus was titrated by plaque assay on L cells at 31 and 39°C (33). No revertants were detected in the stocks of the *ts* mutants used.

Viral protein concentrations were determined by the method of Bradford (10) with reagents from BioRad Laboratories, Richmond, Calif., and using bovine plasma gamma globulin as a standard.

Transcriptase assays. Transcriptase assays were carried out as described previously by Hunt and Wagner (20). All assays contained 700 μM ATP, 700 μM CTP, and 700 μM GTP, 60 μM UTP, plus 10 μCi of [^{3}H]UTP, or 100 μCi of [^{3}H]ATP, or 5 to 10 μCi of [α-^{32}P]UTP, per ml, as required. *S*-adenosylmethionine (SAM; 20 μM) was included in all assays unless otherwise stated. In assays for methylation activity, reactions contained 10 μM SAM (5 Ci/mmol). Transcription assays contained 150 μg of viral protein per ml unless otherwise stated.

To determine the picomoles of each isotopically labeled compound incorporated, the counts per minute per picomole were determined directly by counting a sample of the reaction mix by liquid scintillation spectrophotometry.

Incorporation of radioactivity into ice-cold trichloroacetic acid-precipitable material was assayed as previously described (19), except that the nitrocellulose filters were treated with 0.8 ml of 0.05 M KOH for 20 min, and 8 ml of Ready-Solv HP (Beckman Instruments, Inc., Fullerton, Calif.) was added before liquid scintillation spectrophotometry.

RNA purification and characterization. Transcription reaction mixes were centrifuged at 125,000 × *g* for 150 min in an SW50.1 rotor to remove template ribonucleoprotein. The supernatant fractions were adjusted to 0.15% sodium dodecyl sulfate (SDS) and 300 μg of yeast RNA per ml, extracted with phenol (saturated with 0.15 M NaCl–1 mM EDTA–10 mM Tris-hydrochloride [pH 8.3]), and then desalted on a Sephadex G-50 column equilibrated with 0.05 M ammonium acetate; the RNA-containing fractions were lyophilized and dissolved in water. Unless specifically stated, RNAs were not fractionated on oligodeoxythymidylic acid [oligo(dT)] cellulose.

RNA was subjected to electrophoresis on 2% acrylamide–0.5% agarose composite gels (13). Samples were loaded in a buffer containing 8 M urea–10 mM Tris-hydrochloride (pH 7.4)–0.2% SDS, bromophenol blue, and xylene cyanol FF (RNA sample buffer). [^{14}C]uridine-labeled BHK-21 cell ribosomal RNAs were used as markers. Gels were sliced, digested, and counted by liquid scintillation counting as described previously by Emerson et al. (13).

The proportion of [^{3}H]AMP in product RNA present as polyadenylic acid [poly(A)] was determined by digestion with pancreatic RNase (Worthington Diagnostics, Freehold, N.J.) and T_1 RNase. RNA was doubly labeled with [^{3}H]AMP and [α-^{32}P]UMP and dissolved in a solution of 0.15 M NaCl, 40 μg of carrier RNA per ml, and 10 mM Tris-hydrochloride (pH 7.4). Samples were either not incubated; incubated at 37°C for 1 h (to check for endogenous nucleases); incubated at 37°C for 1 h with 509 μg of pancreatic RNase per ml and 5,217 U of T_1 RNase per ml; or incubated with RNases followed by incubation with 0.3 M NaOH at 37°C for 3 h (to determine the true filter background). All samples were treated in duplicate. Ice-cold trichloroacetic acid-precipitable counts were determined by filtration through 0.45-μm cellulose acetate filters as described above. The percentage of poly(A) is expressed as the RNAse-resistant counts (corrected for the filter background)/unincubated total counts × 100. No evidence for endogenous nucleases was found. In all cases, the [^{32}P]UMP counts were more than 99.5% RNase sensitive, indicating that little, if any, double-stranded RNA was present.

To determine the nature of the 5′ end of methylated RNA, RNA labeled with [^{3}H]SAM was digested with penicillium (P1) nuclease (Sigma Chemical Co., St. Louis, Mo.) and bacterial alkaline phosphatase (Sigma Chemical Co.) and chromatographed on thin-layer cellulose plates (Cel400 M-N; Brinkmann Instruments, Inc., Westbury, N.Y.) in isobutyric acid–0.5 M NH_4OH, 5:3 by volume (9, 22). Cap structures were eluted from the chromatograms with water, digested with nucleotide pyrophosphatase (from *Crotalus adamanteus*; Sigma Chemical Co.) and bacterial alkaline phosphatase, and subjected to thin-layer chromatography in isopropanol-water-NH_4OH, 7:2:1 by volume (9, 22). Enzyme concentrations and buffers were as described previously by Lavi and Shatkin (22), except that the addition of alkaline phosphatase contributed 10 mM Tris-hydrochloride (pH 8.0) to the reactions. All enzyme digestions were performed at 37°C for 30 min. Marker compounds were located by UV illumination. Radioactive compounds were located by slicing the chromatogram, treating with 0.05 M KOH and Ready-Solv HP, and counting as described above. Marker cap structures and 7-methyl guanosine were from P-L Biochemicals, Inc., (Milwaukee, Wis.); other nucleoside markers were obtained from Sigma Chemical Co. All thin-layer plates were prerun with water before use.

Oligo(dT) cellulose column chromatography. Oligo(dT) cellulose column chromatography was performed by a modification of the method of Aviv and Leder (3), using 0.5 M NaCl–0.1% SDS–1 mM EDTA–10 mM Tris-hydrochloride (pH 7.4) at room temperature as binding buffer and 1 mM EDTA at 37°C (mRNAs) or 50°C [poly(A) sizing experiments] as elution buffer. The elevated temperature was found to be necessary to obtain complete elution of the *ts*G16(I) products. RNAs were desalted by Sephadex G-50 chromatography and lyophilized.

Sizing of poly(A) regions. Viral poly(A)$^+$ RNA, labeled with [α-^{32}P]UMP and [^{3}H]AMP and containing 25 to 60 μg of carrier yeast RNA, was dissolved in 100 μl of 0.3 M NaCl–10 mM Tris-hydrochloride (pH 7.4) and treated with 120 U of T_1 RNase (Bethesda Research Laboratories, Gaithersburg, Md.) or 5 μg of pancreatic RNase (Worthington), or both enzymes, for 30 min at 37°C. Samples were then adjusted to 0.2% SDS, 20 mM EDTA, and 140 μg of yeast RNA per ml and extracted with 3.5 volumes of phenol-chloroform (1:1), and the poly(A)$^+$ RNA was purified by oligo(dT) chromatography, ethanol precipitated, and dissolved in RNA sample buffer.

Before use, the RNase solutions supplied by the manufacturers were diluted 10-fold with 0.3 M NaCl–

10 mM Tris-hydrochloride (pH 7.4) and heated at 95°C for 5 min.

Translation. Viral RNAs were translated in an in vitro reticulocyte lysate translation system (Amersham Corp., Arlington Heights, Ill.), using [^{35}S]methionine to label the products and under conditions in which the amount of RNA was rate limiting. All translation assays contained 300 μM *S*-adenosylhomocysteine (SAH) to prevent methylation of RNAs. After incubation, 11-μl reaction mixtures were treated with 32.5 μg of pancreatic RNAse for 15 min at 31°C, and the proteins were precipitated by the addition of 50 volumes of 10:1 acetone-ammonium hydroxide for 20 min at room temperature. After centrifugation for 5 min in an Eppendorf centrifuge, the pellets were dissolved in 8 M urea–10 mM Tris-hydrochloride (pH 7.4)–1% SDS–1% β-mercaptoethanol containing bromophenol blue and heated at 100°C for 5 min before polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10 or 12.5% gels by the method of Laemmli (21), except that the bisacrylamide concentration was adjusted according to the formula: acrylamide (g/100 ml) = 1,300/bisacrylamide (mg/100 ml). After electrophoresis, gels were subjected to fluorography with En^{3}Hance (New England Nuclear Corp., Boston, Mass.).

Chemicals. [5,6-^{3}H]UTP (24 to 40 Ci/mmol) and [2,8-^{3}H]ATP (25 to 50 Ci/mmol) were from ICN Radioisotopes, Irvine, Calif. *S*-adenosyl-L(*methyl*-^{3}H]methionine (5 to 15 Ci/mmol), [α-^{32}P]UTP (more than 400 Ci/mmol), and L-[^{35}S]methionine (more than 800 Ci/mmol) were obtained from Amersham Corp. Oligo(dT) cellulose was bought from Collaborative Research, Inc., Waltham, Mass. (T3 grade) or Bethesda Research Laboratories. Human placental RNase inhibitor was obtained from Bethesda Research Laboratories or Biotech Inc. Madison, Wis. (RNasin). Nitrocellulose filters (45 μm; BA 85) were from Schleicher & Schuell Co., Keene, N.H.

RESULTS

In vitro poly(A) synthesis by *ts*G16(I). In the course of a survey of *ts* mutants of VSV (Indiana) to find those with aberrant RNA synthesis in vitro, I found that the RNA synthesized by *ts*G16(I) contained a higher percentage of [^{3}H]AMP in poly(A) than did *wt* virus (Table 1). Such a high proportion of poly(A) in the product was not a general feature of Glasgow *ts* mutants nor was it common to all group I mutants (Table 1). *ts*G13(I), *ts*G21(II), and *ts*G41(IV) showed proportions of poly(A) similar to that shown by *wt* VSV (which ranged from 10.6 to 19.1% in four experiments), but *ts*G16(I) product RNA had approximately two times (or more) the percentage of [^{3}H]AMP present as poly(A) than did any of the other viruses examined in this experiment.

Temperature sensitivity of polyadenylation by *ts*G16(I). Since *ts*G16(I) possesses a temperature-sensitive transcriptase activity (19, 28), it was of interest to determine whether the high degree of polyadenylation by this mutant showed any temperature dependence. Accordingly, I assayed the poly(A) content of the product RNA of *wt* and *ts*G16(I) transcription reactions performed at 27, 31, or 35°C (39°C, the nonpermissive temperature for *ts*G16(I), could not be used for this experiment since very little RNA is made at 39°C, whether UMP or AMP is used to monitor RNA synthesis [19, 28; D. M. Hunt, unpublished data]). The results (Table 2) indicated that polyadenylation by *ts*G16(I) was abnormal at 27, 31, and 35°C.

TABLE 1. Percentage of AMP residues present as poly(A) in in vitro transcription products of *wt* VSV and *ts* mutants

Virus[a]	% of AMP residues present as poly(A)[b]
wt	13.6
*ts*G13(I)	18.0
*ts*G16(I)	36.4
*ts*G21(II)	18.6
*ts*G41(IV)	12.6

[a] RNA was made in vitro at 31°C using purified virions and a 2-h incubation period.

[b] Poly(A) content was assayed by determining the percentage of [^{3}H]AMP counts in doubly labeled RNA ([^{3}H]AMP, [^{32}P]UMP) resistant to digestion with T_1 and pancreatic RNases, as measured by ice-cold trichloroacetic acid precipitation. In all cases, RNase digestion rendered more than 99.5% of the UMP residues soluble in ice-cold trichloroacetic acid, suggesting that little or no double-stranded RNA was present in the sample.

In vitro translation of total product RNA. One question which arises is whether the RNA made in vitro by *ts*G16(I) was abnormal in ways other than the high content of poly(A). I therefore used total product RNA from in vitro transcription reactions to program an in vitro reticulocyte lysate cell-free translation system under conditions in which the amount of RNA was rate limiting. Equal amounts of mRNA (as measured by [^{32}P]UMP labeling) were used to program translation at 101 or 190 mM K^+. In this system, methylated VSV mRNA translated with almost equal efficiency at both K^+ concentrations, whereas unmethylated VSV mRNA translated poorly at 190 mM K^+, although it translated approximately as well as methylated RNA at 101 mM K^+ (D. M. Hunt and W. Y. Kao, unpublished data). This is consistent with observations made using globin mRNA (11, 34). All translations were done in the presence of 300 μM SAH to inhibit further methylation of RNA.

The results (Fig. 1) showed that RNA synthesized by *ts*G16(I) in vitro was functional as mRNA. *ts*G16(I) RNA was translated (on a

TABLE 2. Effect of temperature on percentage of AMP residues present as poly(A) in in vitro transcripts

Virus[a]	% AMP residues present as poly(A) in RNA made in vitro at:[b]		
	27°C	31°C	35°C
wt			
A	19.4	13.3	15.8
B	ND[c]	13.6	11.2
*ts*G16(I)			
A	51.2	54.1	60.5
B	ND	36.4	41.8

[a] A and B refer to independent experiments.
[b] Poly(A) content was assayed by determining the percentage of [^{3}H]AMP-labeled RNA resistant to digestion with pancreatic and T_1 RNases.
[c] ND, Not done.

qualitative basis) almost equally well whether it was made at 27, 31, or 35°C and was translated as well as *wt* RNA made at 31°C. The same products (predominantly N, NS, and M proteins) were seen. Furthermore, raising the K^+ concentration from 101 to 190 mM had little effect on translation, suggesting that the mRNA was at least partially methylated.

Incorporation of methyl groups into RNA. To see if the extents of methylation of *wt* and *ts*G16(I) RNA were similar, I made RNA in in vitro transcription reactions using *S*-adenosyl-[*methyl*-^{3}H]methionine and [α-^{32}P]UTP to label the product RNA. I determined the picomoles of ^{3}H-methyl group incorporated relative to the picomoles of [α-^{32}P]UMP incorporated as a function of time by determination of the ice-cold trichoroacetic acid-precipitable counts of each isotope incorporated. Results (Table 3) indicate that, per 400 pmol of UMP incorporated, a similar number of methyl groups were incorporated by *wt* virus at 31°C as were incorporated by *ts*G16(I) at 31°C. At 35°C, methylation by *ts*G16(I) was somewhat less than that by *wt* virus. All further experiments were performed with RNA made in vitro at 31°C.

To determine whether *ts*G16(I) RNA made in vitro did indeed contain the 5′ terminal sequence m^{7}G(5′)ppp(5′)Am present at the 5′ terminus of *wt* mRNA made in vitro (2), I digested [^{3}H]SAM-labeled RNA with P1 nuclease and bacterial alkaline phosphatase and examined the products by thin-layer chromatography (Fig. 2A and B). Two peaks were seen with both *wt* and *ts*G16(I) RNA. The major peak (II, Fig. 2A and B) cochromatographed with m^{7}G(5′)ppp(5′)Am. The minor peak (I, Fig. 2A and B) cochromatographed with G(5′)ppp(5′)Am and m^{7}G(5′)ppp(5′)A. The ratio of peak I to peak II ranged from 0.12 to 0.16:1 for *wt* RNA (three determinations) and 0.18 to 0.19:1 for *ts*G16(I) RNA (three determinations). To characterize these structures further, they were eluted, digested with nucleotide pyrophosphatase and bacterial alkaline phosphatase, and again subjected to thin-layer chromatography. Digestion of peak II from *wt* or *ts*G16(I) RNA gave rise to two major peaks of radioactivity which comigrated with m^{7}G and Am and were present in approximately equal amounts (Fig. 2C and D), as would be expected if peak II material were m^{7}G(5′)ppp(5′)Am. Digestion of peak I material from either *wt* or *ts*G16(I) RNA gave rise to a single methylated product which comigrated with Am (Fig. 2E and F). This would suggest that the material in peak I was G(5′)ppp(5′)Am and not m^{7}G(5′)ppp(5′)A. [Testa and Banerjee [29] have reported that G(5′)ppp(5′)Am is pres-

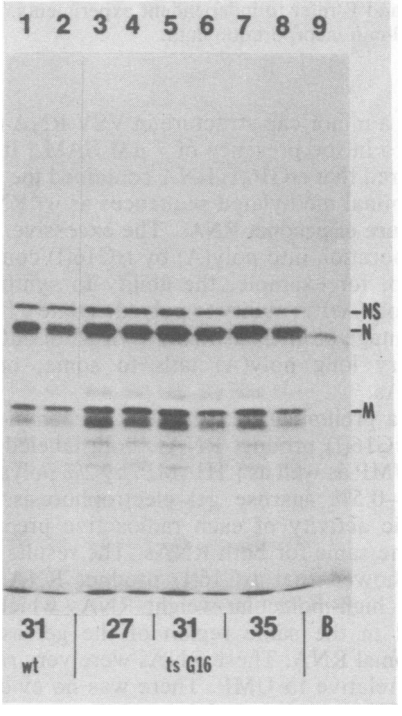

FIG. 1. In vitro reticulocyte lysate cell-free translation system products of *wt* and *ts*G16(I) RNA made at various temperatures. The reticulocyte lysate system was programmed with RNA (containing 11 pmol of UMP) made in vitro by *wt* virus at 31°C (lanes 1 and 2) or by *ts*G16(I) at 27°C (lanes 3 and 4), 31°C (lanes 5 and 6), or 35°C (lanes 7 and 8); or no RNA was added to the lysate (lane 9). Translation reactions were all carried out at 31°C for 90 min in the presence of 300 μM SAH and either 101 mM K^+ (lanes 1, 3, 5, and 7) or 190 mM K^+ (lanes 2, 4, 6, 8, and 9). Potassium concentrations were adjusted by the addition of potassium acetate. Products were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. The position of VSV marker proteins (stained by Coomassie blue) is indicated at the right-hand side.

TABLE 3. Methylation of RNA synthesized in vitro by *wt* virus or *ts*G16(I)[a]

Virus[b]	pmol of methyl group incorporated per 400 pmol of UMP incorporated in RNA synthesized in vitro at:[c]	
	31°C	35°C
wt		
A	1.9	1.8
B	2.1	1.9
*ts*G16(I)		
A	1.9	1.4
B	1.6	1.4

[a] Picomoles of methyl group incorporated per 400 pmol of UMP incorporated into ice-cold trichloroacetic acid-precipitable material were determined by using transcription reaction mixes containing [^{3}H]SAM and [α-^{32}P]UTP.

[b] A and B refer to independent experiments.

[c] 120-min incorporation data.

ent as a minor cap structure in VSV RNA made in vitro in the presence of 7 μM SAM.] It thus appeared that *ts*G16(I) RNA contained the same 5′ terminal methylated sequences as *wt* RNA.

Nature of product RNAs. The excessive AMP incorporation into poly(A) by *ts*G16(I) could be due to, for example, the ability to synthesize free poly(A), an ability to polyadenylate a higher percentage of mRNAs than *wt* virus, or addition of very long poly(A) tails to some, or all, mRNAs.

As a preliminary experiment, I examined *wt* and *ts*G16(I) product RNAs, both labeled with [^{32}P]UMP as well as [^{3}H]AMP, by 2% polyacrylamide–0.5% agarose gel electrophoresis. The specific activity of each radioactive precursor was the same for both RNAs. The results (Fig. 3B) showed that *ts*G16(I) product RNA contained high-molecular-weight RNAs which migrated in the same region of the gel as 28S ribosomal RNA. These RNAs were very rich in AMP relative to UMP. There was no evidence for any *wt* species so rich in AMP versus UMP and, furthermore, *wt* product contained very little RNA which migrated in the 28S region of the gel (Fig. 3A).

To elucidate further the nature of the product RNA, I fractionated it into poly(A)$^+$ and poly(A)$^-$ species by oligo(dT) cellulose chromatography. For these experiments, I included human placental RNase inhibitor (50 U/ml) in the transcription reactions to protect against any slight contamination of the virion preparations by ribonuclease. The poly(A)$^+$ and poly(A)$^-$ RNAs were subjected to polyacrylamide-agarose gel electrophoresis (Fig. 4). The poly(A)$^-$ RNAs synthesized by either *wt* virus (Fig. 4C) or *ts*G16(I) (Fig. 4D) had a similar size distribution and, not surprisingly, a similar ratio of AMP-to-UMP incorporation. However, the poly(A)$^+$ RNAs synthesized by each virus were very different. Three peaks of *wt* poly(A)$^+$ RNA were observed (Fig. 4A), with molecular weights of approximately 0.9×10^6 (Fig. 4A, a), 0.4×10^6 (Fig. 4A, b), and 0.3×10^6 (Fig. 4A, c). *ts*G16(I) poly(A)$^+$ RNA (Fig. 4B) was mainly of higher molecular weight, with much of the RNA having an apparent molecular weight equal to or more than that of 28S ribosomal RNA (1.65×10^6 [23]). There were no obvious 0.9×10^6- or 0.3×10^6 to 0.4×10^6-molecular-weight RNA species in the *ts*G16(I) product. Furthermore, all size classes of *ts*G16(I) RNA were enriched for AMP relative to UMP as judged by the ratio of AMP-to-UMP counts compared with that seen with *wt* poly(A)$^+$ RNA.

Translation of poly(A)$^+$ RNA. Since the poly(A)$^+$ RNA made by *ts*G16(I) was so large, I wished to know whether this RNA could code for proteins and, if so, whether it had the same coding potential as *wt* poly(A)$^+$ RNA in the reticulocyte cell-free translation system. Equal amounts of UMP-labeled RNA were used to program the reticulocyte lysate, and the results (Fig. 5) showed that poly(A)$^+$ RNA from either *ts*G16(I) or *wt* in vitro transcription reactions coded for the synthesis of the same viral proteins in qualitatively similar amounts at both 101 and 190 mM K^+. Furthermore, there was no obvious difference between the proteins coded for by unfractionated RNA or poly(A)$^+$ RNA.

Size of poly(A). The presence of an unusually high-molecular-weight, AMP-rich, poly(A)$^+$ mRNA in *ts*G16(I) in vitro-product RNA could be due to addition of abnormally long poly(A) tails to otherwise normal mRNAs. To obtain an estimate of the length of the poly(A) in poly(A)$^+$ RNA from either *wt* or *ts*G16(I) in vitro transcription reactions, I digested poly(A)$^+$ RNA made at 31°C and labeled with both [^{3}H]AMP and [^{32}P]UMP with either T_1 RNase, pancreatic RNase, or both RNases. Poly(A) stretches should be left intact by such treatment, but the rest of the VSV mRNAs should be digested away from the poly(A) stretches. Poly(A)$^+$ RNA was isolated from the RNase digestion reactions and subjected to 2% acrylamide–0.5% agarose gel electrophoresis. The results (Fig. 6) showed that RNase digestion was complete, as assayed by the absence of [^{32}P]UMP counts associated with the poly(A)$^+$ RNA. After digestion with T_1 RNase, pancreatic RNase, or both RNases, the poly(A)$^+$ RNA from *wt* RNA had a mobility equivalent to that of an RNA of approximately 160 to 320 nucleotides (region of bar, Fig. 6A), 160 to 320 nucleotides (bar, Fig. 6B), or 150 to 200 nucleotides (bar, Fig. 6C), respectively.

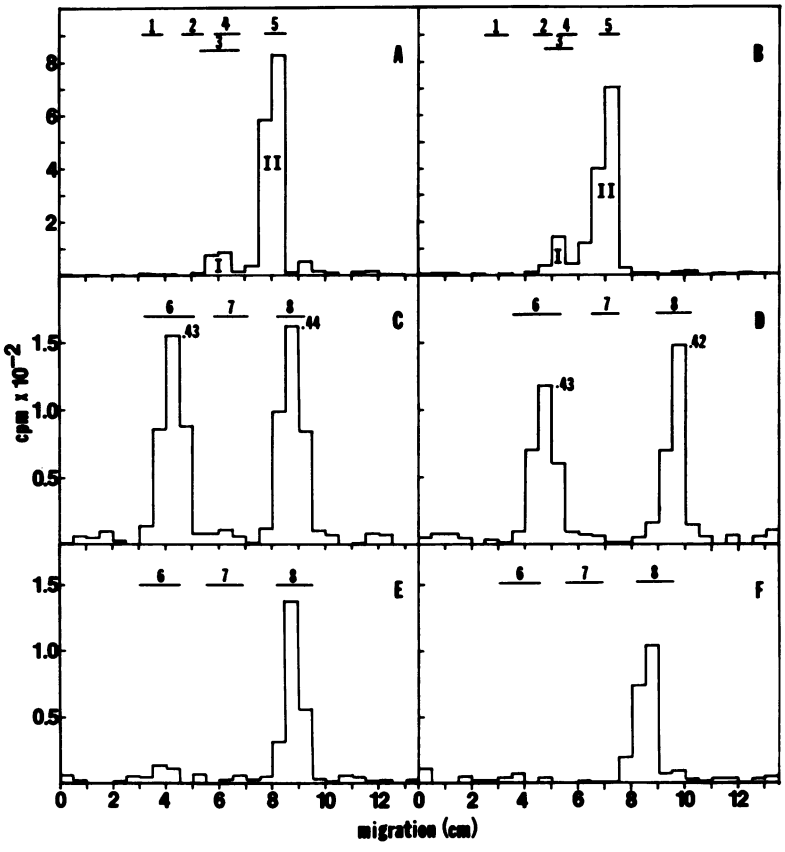

FIG. 2. Characterization of methylated cap structures present in RNA made in vitro at 31°C by *wt* virus or *ts*G16(I). *wt* (A, C, E) or *ts*G16(I) (B, D, F) RNA labeled with [^{3}H]SAM was treated with P1 nuclease and alkaline phosphatase and analyzed by thin-layer chromatography, using isobutyric acid–0.5 M NH_4OH, 5:3 by volume (A, B). The material in peak II or peak I of these chromatograms was eluted, digested with nucleotide pyrophosphatase and alkaline phosphatase, and chromatographed with isopropanol-water-NH_4OH, 7:2:1 by volume. C and D show peak II digestion products of *wt* virus and *ts*G16(I), respectively; E and F show peak I digestion products. The following markers were used: 1, $m^7G(5')ppp(5')G$; 2, $m^7G(5')ppp(5')Gm$; 3, $G(5')ppp(5')Am$; 4, $m^7G(5')ppp(5')A$; 5, $m^7G(5')ppp(5')Am$; 6, m^7G; 7, Gm; 8, Am.

With *ts*G16(I) RNA, however, the products were considerably larger. Digestion with T_1 RNase, pancreatic RNase, or both RNases gave products with a mobility equivalent to that of an RNA of approximately 1,530 to 2,360 nucleotides, 250 to 650 nucleotides, and 230 to 490 nucleotides, respectively (bars in Fig. 6D, E, and F).

Transcription in absence of methyl donor. Rose et al. (27) have reported that VSV mRNAs made in the presence of 1 mM SAH have long poly(A) tracts. To see if *ts*G16(I) were synthesizing unusually large amounts of poly(A) because the mutant was exquisitely sensitive to SAH which might be present as a contaminant in the SAM used, transcription reactions were carried out in the presence or absence of SAM, and the relative incorporation of [^{3}H]AMP and [^{32}P]UMP residues into *wt* or *ts*G16(I) product RNA was measured.

The results (Table 4) showed that with either virus, neither the total incorporation of nucleotide nor the ratio of AMP-to-UMP residues incorporated was significantly affected by omission of SAM from transcription mixes.

DISCUSSION

In vitro RNA synthesis by virions of our stocks of *ts*G16(I) was aberrant compared with in vitro RNA synthesis by *wt* virus. The initial observation was that the ratio of AMP-to-UMP incorporation in an in vitro transcription system was much higher with *ts*G16(I) than with *wt* virus (e.g., Table 4). The percentage of AMP residues present as poly(A) was determined by

TABLE 4. Effect of presence or absence of SAM during transcription on ratio of AMP to UMP residues incorporated by *wt* virus or *ts*G16(I)[a]

Virus[b]	Presence (+) or absence (−) of SAM[c]	pmol incorporated[d]		Ratio (AMP/UMP)
		UMP	AMP	
wt	−	224	344	1.54
	+	227	347	1.53
*ts*G16(I)	−	147	476	3.24
	+	156	490	3.14

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

[b] Assays were performed at a viral protein concentration of 100 μg/ml.

[c] SAM, when present, was 20 μM.

[d] 120-min incorporation data.

digestion with pancreatic and T_1 RNases, which should result in cleavage at C, G, and U, but not A, residues. RNA was doubly labeled with [^{3}H]AMP and [^{32}P]UMP so that the proportion of AMP residues resistant to RNase digestion could be determined while simultaneously ensuring that digestion was complete and that no significant amount of double-stranded RNA was present. The results (Table 1) showed that *ts*G16(I) RNA made in vitro at 31°C contained a considerably higher proportion of AMP residues in poly(A) than did RNA from *wt* virus or *ts*G13(I), *ts*G21(II), or *ts*G41(IV). Although *ts*G16(I) has been shown to contain a temperature-sensitive transcriptase activity due to a defective L protein (18), lowering the temperature of transcription to 27°C or raising it to 35°C gave no clear evidence that the abnormal polyadenylation was due to a temperature-sensitive component (Table 2). I have, so far, had no success in obtaining revertants from my stocks of *ts*G16(I) to see if the *ts* phenotype and the aberrant polyadenylation activity are due to the same mutation. To identify the moiety involved in abnormal polyadenylation, I am using protein fractionation-transcription reconstitution experiments similar to those previously described (18).

The question arises as to whether the RNA made by *ts*G16(I) in vitro is abnormal in other aspects. RNA from either *ts*G16(I) or *wt* in vitro transcription reactions was used to program a reticulocyte cell-free translation system. Both RNAs were approximately equally efficient when equivalent amounts of RNA were added (as measured by UMP residues), and the same three viral proteins, NS, N, and M, were made in similar ratios (Fig. 1). Since translation of *ts*G16(I) product RNA was not markedly affected by raising the potassium ion concentration from 101 to 190 mM, it seemed probable that the RNA was at least partially methylated. This was confirmed by measuring the amount of incorporation of methyl group from SAM into RNA relative to the amount of UMP incorporation. At 31°C, the extent of methylation of *ts*G16(I) product was 76 to 100% of that of *wt* virus, and at 35°C it was 73 to 78% (Table 3). Direct determination of the 5′ terminal sequence of [^{3}H]SAM-labeled RNA showed that both *wt* and *ts*G16(I) RNAs were capped and methylated (Fig. 2). The properties of the major methylated cap structure in both RNAs were consistent with the structure m^{7}G(5′)ppp(5′)Am. Both RNAs contained a minor methylated cap structure. This was present in a slightly higher ratio to the major cap structure in *ts*G16(I) RNA (0.18 to 0.19:1) than in *wt* RNA (0.12 to 0.16:1). The properties of the minor methylated cap structure suggested that it was G(5′)ppp(5′)Am. These cap structures are consistent with previous reports of the nature of the 5′ methylated caps in *wt* VSV RNA made in vitro (2, 29). It therefore appeared that guanylyltransferase and both methyltransferase activities (29) were functional in *ts*G16(I) and that *ts*G16(I) RNA made in vitro at 31°C was probably normally modified at the 5′ end.

*ts*G16(I) RNA made in vitro differed from *wt* RNA in size. *ts*G16(I) made a species of RNA which migrated in the same region of the gel as 28S ribosomal RNA and which was rich in AMP relative to UMP (Fig. 3). When the poly(A)$^{+}$ RNA fraction was purified from *ts*G16(I) product RNA, it was found to be predominantly higher in molecular weight and richer in AMP-to-UMP residues than *wt* poly(A)$^{+}$ RNA (Fig. 4). There was little evidence for the presence in *ts*G16(I) poly(A)$^{+}$ RNA of any RNAs corresponding to *wt* RNA species in terms of either molecular weight or AMP-to-UMP ratio. However, the poly(A)$^{+}$ RNA from *ts*G16(I) coded for the same proteins, with approximately the same efficiency (on a per picomole of UMP residues basis), as did the poly(A)$^{+}$ RNA from *wt* virus. These data would be consistent with the hypothesis that all poly(A)$^{+}$ mRNAs made in vitro by *ts*G16(I) contain abnormally long tracts of poly(A), resulting in their apparent high molecular weight. Further evidence to support this comes from RNase digestion experiments. Digestion of *ts*G16(I) poly(A)$^{+}$ RNA with T_1 ribonuclease followed by oligo(dT) cellulose column chromatography gave evidence for long poly(A) tracts, a large proportion of which had a mobility equivalent to an RNA of 1,500 to 2,400 nucleotides (bar in Fig. 6D) and with some material of even higher apparent molecular weight. Poly(A)

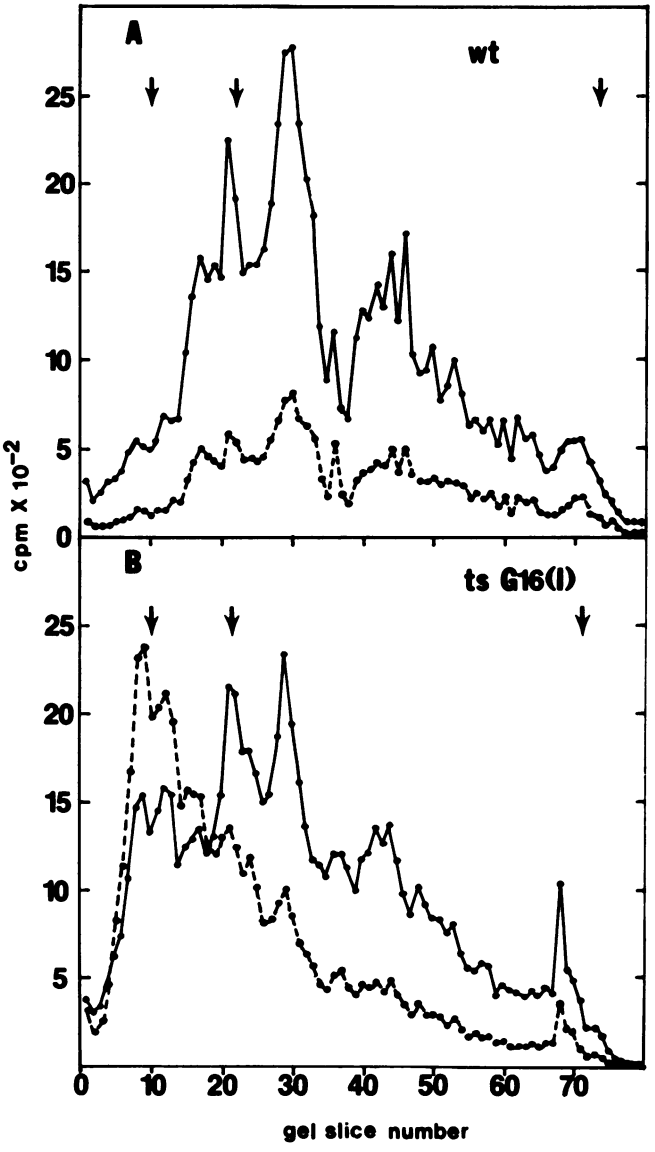

FIG. 3. 2% polyacrylamide–0.5% agarose gel electrophoresis of RNA synthesized in vitro by purified virions of *wt* virus (A) or *ts*G16(I) (B). RNA was synthesized in vitro in the presence of both [α-^{32}P]UTP and [^{3}H]ATP. The specific activities of the precursors were the same in *wt* and *ts*G16(I) transcription mixes. Product RNA was purified and subjected to electrophoresis on 2% acrylamide–0.5% agarose gels, which were sliced, digested, and counted by dual-channel liquid scintillation spectrometry. Symbols: ●, [^{32}P]UMP; ○, [^{3}H]AMP. Arrows indicate the position of 28, 18, and 4S BHK-21 ribosomal markers run on a parallel gel. Direction of electrophoresis was from left to right.

tracts in *wt* product RNA were considerably smaller (approximately 160 to 320 nucleotides; Fig. 6A). Digestion with pancreatic RNase gave shorter poly(A)$^{+}$ products than did digestion with T_1 RNase, although these products were still larger for *ts*G16(I) RNA than for *wt* RNA. We do not know whether this indicates cleavage at A residues under our digestion conditions, or whether the poly(A) tracts contain occasional C or U residues. There is evidence for a low frequency of nonbase-specific cleavage by both T_1 and pancreatic RNases (26, 30). It also is possible that the poly(A) tracts may be even larger than suggested by Fig. 6D if T_1 RNase

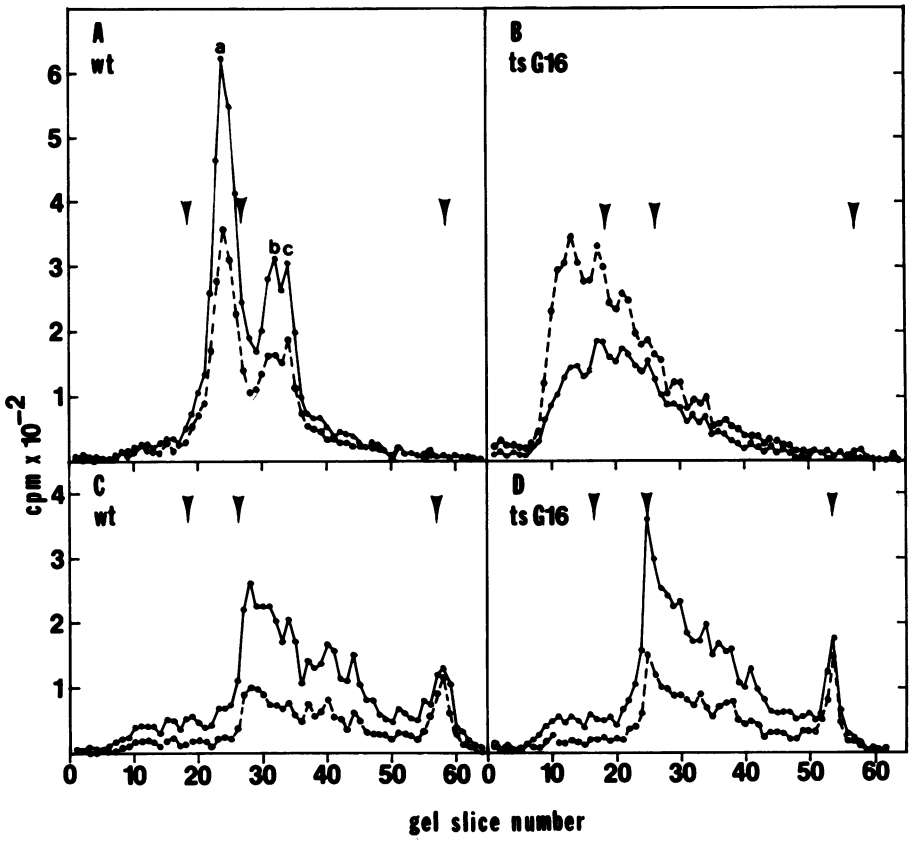

FIG. 4. 2% polyacrylamide–0.5% agarose gel electrophoresis of poly(A)$^+$ and poly(A)$^-$ RNA synthesized in vitro by *wt* virus or *ts*G16(I). RNA was synthesized in vitro at 31°C in the presence of both [α-^{32}P]UTP and [^{3}H]ATP. The specific activities of the precursors were the same in *wt* and *ts*G16(I) transcription mixes. Product RNA was separated into poly(A)$^+$ and poly(A)$^-$ RNAs by oligo(dT) cellulose chromatography and subjected to 2% polyacrylamide–0.5% agarose gel electrophoresis. A, poly(A)$^+$ *wt* RNA; B, poly(A)$^+$ *ts*G16(I) RNA; C, poly(A)$^-$ *wt* RNA; D, poly(A)$^-$ *ts*G16(I) RNA. Symbols: ●, [^{32}P]UMP; ○, [^{3}H]AMP. Arrows indicate the position of 28, 18, and 4S RNAs on a gel run in parallel.

cleaves at A residues under our conditions, or if the tracts contain infrequent G residues. Data to suggest the poly(A) tracts may be longer than 1,500 to 2,400 nucleotides comes from observations that the N, NS, and M mRNA transcripts are approximately 800 to 1,330 nucleotides long (25). Even 2,400 A residues would not result in a molecular weight as high as 28S RNA (ca. 5,000 nucleotides long), and yet much of the poly(A)$^+$ *ts*G16(I) RNA is larger than 28S RNA (Fig. 4B).

The presence of intervening poly(A) sequences in polycistronic transcripts of VSV has been reported (16, 17). I do not know if a similar phenomenon may be occurring in *ts*G16(I) transcripts and contributing to the high molecular weight of the poly(A)$^+$ transcripts. I am currently investigating this possibility.

Long poly(A) tracts are made by VSV in the presence of SAH (27). The possible presence of SAH in our SAM solutions is unlikely to explain our data, since *wt* virus does not exhibit the abnormal polyadenylation pattern and, second, since omission of SAM from transcription reactions does not affect the AMP-to-UMP ratio in the RNA made by either *wt* or *ts*G16(I) reactions (Table 4).

It is unlikely that the aberrant RNA made by *ts*G16(I) is due to a change in the polyadenylation signal in the nucleotide sequence of the template since all mRNAs assayed for (N, NS, and M) were present as high-molecular-weight, A-rich RNAs, and it seems improbable that three independent mutations having the same effect have occurred. Furthermore, preliminary experiments in which *ts*G16(I) or *wt* virus was fractionated into template- and enzyme-contain-

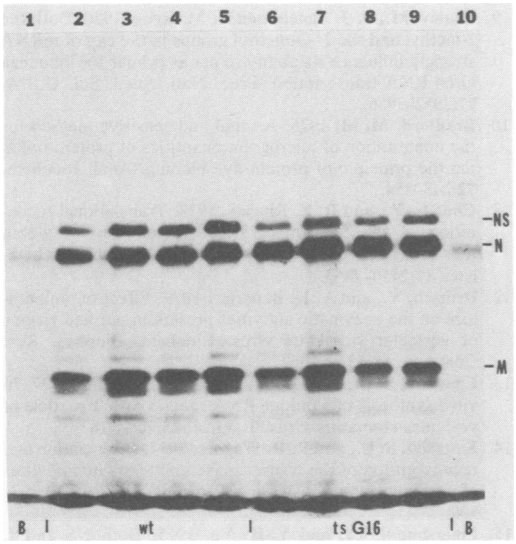

FIG. 5. In vitro translation products in a cell-free system programmed by unfractionated or poly(A)$^+$ RNA from *wt* or *ts*G16(I) transcription reactions. RNA was made at 31°C in vitro in the presence of human placental RNase inhibitor. The product RNA was purified and either assayed directly (lanes 2, 3, 6, and 7) or after selection of poly(A)$^+$ RNA by oligo(dT) cellulose chromatography (lanes 4, 5, 8, and 9). The in vitro reticulocyte translation system was programmed with viral RNA containing 11 pmol of UMP (lanes 2–9) or no RNA (lanes 1 and 10) in the presence of 300 μM SAH and either 101 mM K^+ (lanes 1, 2, 4, 6, and 8) or 190 mM K^+ (lanes 3, 5, 7, 9, and 10). Translation products were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. The position of VSV marker proteins is indicated at the right-hand side.

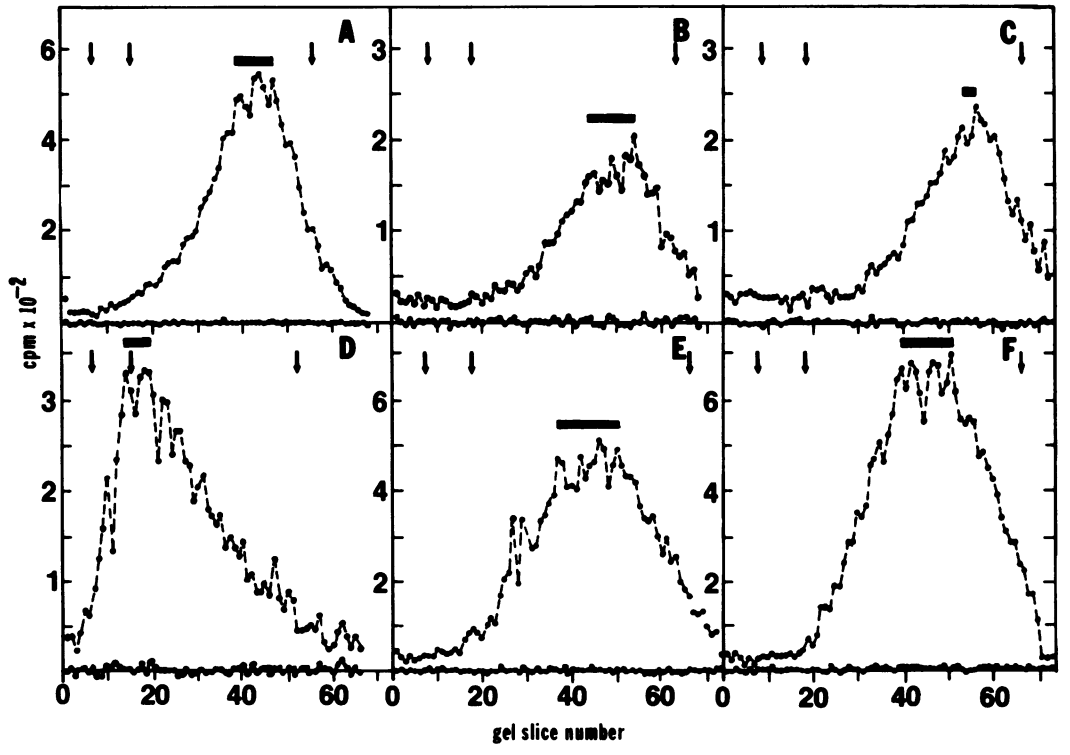

FIG. 6. Length of poly(A)$^+$ RNA from *wt* or *ts*G16(I) RNA after RNase digestion. Poly(A)$^+$ RNA made at 31°C in vitro, in the presence of human placental RNAse inhibitor, by *wt* virus (A, B, C) or by *ts*G16(I) (D, E, F) was digested with T_1 RNase (A, D), pancreatic RNase (B, E), or both RNases (C, F). The poly(A)$^+$ RNA was isolated and subjected to electrophoresis on 2% acrylamide–0.5% agarose gels. The solid arrows indicate the positions of 28, 18, and 4S marker RNAs on parallel gels. Symbols: ●, [^{32}P]UMP counts per minute; ○, [^{3}H]AMP counts per minute.

ing fractions and recombined in homologous or heterologous reconstitution experiments of the type previously described (19) indicate that the aberrant polyadenylation in *ts*G16(I) transcription assays is due to a moiety in the enzyme-containing fraction (D. M. Hunt, unpublished data).

It therefore appears that a virally coded protein can affect polyadenylation. One possibility is that the poly(A) polymerase itself is a virally coded protein and is altered in *ts*G16(I). Alternatively, the poly(A) polymerase could be either a virally coded or a host protein present in virions, and the extent of polyadenylation by this enzyme could be controlled by a separate virally coded protein which is altered in *ts*G16(I). Another possibility is that the defect in *ts*G16(I) may not be directly connected with polyadenylation, but rather with initiation. Since transcription by VSV is sequential, it is then possible that the length of poly(A) added to each mRNA is determined, at least partially, by the initiation events at the next gene, the initiation sites for NS, M, G, or L genes being only two nucleotides away from the end of the putative polyadenylation signal of the previous gene (25). These hypotheses are being investigated.

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