JOURNAL OF VIROLOGY, Sept. 1977, p. 708-716

Vol. 23, No. 3
Printed in U.S.A.

In Vitro Synthesis of a Unique RNA Species by a T Particle of Vesicular Stomatitis Virus

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Received for publication 7 April 1977

A T particle of vesicular stomatitis virus, containing most of the *L*-gene region, has been isolated. In vitro, these T particles synthesize exclusively a small adenine-rich RNA that is complementary to the T-particle genome. Partial sequence analysis of this small RNA indicates that it is an RNA of unique sequence with a length of approximately 45 nucleotides.

Vesicular stomatitis virus (VSV) contains a single strand of minus-sense RNA that codes for five proteins, N, NS, M, G, and L (28). The linear arrangement of the genes coding for these proteins in wild-type virus has been shown to be 3'-*N-NS-M-G-(L)*-5' (1, 4). However, when VSV is propagated at a high multiplicity of infection, a new class of virus particles (T particles) is generated that appear shorter than the normal VSV particles (B particles) (17). Characterization of a number of T particles of various sizes has shown that they all contain incomplete genomes and usually are derived from the region of the viral genome coding for the L protein (25). Since T particles are defective for replication, they require the co-replication of B particles to provide missing functions. Also, T particles apparently replicate more quickly than B particles and interfere with their production. The mechanisms of T-particle generation and interference with B-particle replication are not presently understood.

B particles contain an RNA-dependent RNA polymerase that synthesizes mRNA in vitro, using the B-particle nucleocapsid as template (5, 9, 21). This mRNA is capped at the 5' end with ^{7m}G and is polyadenylated at the 3' end (2, 7, 26). Most of the mRNA is synthesized from the region of the genome coding for the N, NS, M, and G proteins; the region of the genome coding for the L protein does not appear to be efficiently transcribed in vitro (9, 16). The in vitro synthesis of RNA by T particles has also been investigated by a number of laboratories (8, 13, 20, 23). Reichmann et al. examined six different T particles and concluded that all T particles reproducibly synthesized a small quantity of adenine-rich RNA that was considerably shorter than the RNA synthesized by B particles (23). The nucleotide and ion requirements for this RNA synthesis were similar to the requirements for transcription by B particles. However, Reichmann et al. were unable to hybridize the T-particle products to virion RNA and thus could not demonstrate that these RNAs were coded by the T-particle template. In contrast, Mori and Howatson reported that certain T-particle preparations synthesized RNA that was hydrogen bonded to T-particle virion RNA, indicating that T particles may function as templates for in vitro synthesis (20).

Reconstitution studies have shown that T particles do contain an RNA-dependent RNA polymerase, since soluble enzyme fractions prepared from either B or T particles were equally effective in promoting synthesis of mRNA from enzyme-deficient B-particle nucleocapsid templates (13). However, only very low levels of RNA synthesis were observed when enzyme fractions from either B or T particles were added to T-particle nucleocapsid templates, suggesting that T-particle templates were defective for RNA synthesis. These observations have led us to reexamine the question of whether T particles actively synthesize RNA in vitro that is template coded. In this paper we report experiments demonstrating that a T particle derived from the *L*-gene region of VSV does serve as template for RNA synthesis; however, the T-particle product is not synthesized from the entire genome, but rather is a unique RNA species approximately 45 nucleotides in length.

MATERIALS AND METHODS

Chemicals and radiochemicals. For the hybridization studies, pancreatic RNase A was obtained from Sigma Chemical Co., St. Louis, Mo., and T1 RNase was obtained from Worthington Biochemicals Corp., Freehold, N.J. α-^{32}P-labeled triphosphates (105 Ci/mmol) and [^{32}P]orthophosphate were purchased from Amersham/Searle, Arlington Heights, Ill. [^{3}H]uridine (27 Ci/mmol), [^{3}H]ATP (17 Ci/mmol), and [^{3}H]UTP (16 Ci/mmol) were obtained

from Schwarz/Mann, Orangeburg, N.Y. Oligo(dT)-cellulose (T2) was from Collaborative Research Inc., Waltham, Mass. Millipore Corp., Bedford, Mass., provided the cellulose acetate filters. Acrylamide and *N,N*-methylenebisacrylamide (Bis) were from Bio-Rad Laboratories, Richmond, Calif., and the agarose was from Marine Colloids, Inc., Springfield, N.J.

Virus. VSV, Indiana serotype, was originally from the U.S. Agricultural Research Center, Beltsville, Md. A clone obtained from Robert R. Wagner and passaged in mouse L cells was recloned and adapted to growth in BHK-21 cells, whereupon it generated the T particle used in these experiments. For the preparation of ^{3}H-labeled VSV RNA, virus was grown in BHK-21 monolayers at 37°C in the presence of 90% BHK-21 medium (containing glutamine) and 10% tryptose phosphate broth with 5 μCi of [^{3}H]uridine per ml. Labeled virions were harvested 16 to 18 h after infection by differential, rate zonal, and equilibrium centrifugation as previously described, except that three rate zonal centrifugations were included (15). A virus clone from the same stock, which did not generate T particles, was used to infect cells for preparation of viral mRNA.

RNA polymerase assays. RNA polymerase assays were carried out as previously described (14). One and one-half volumes of purified B or T virions in 10 mM Tris-hydrochloride (pH 7.4) containing 15% glycerol were mixed with 1 volume of 1× high-salt solubilizer and 2.5 volumes of reaction mix to yield a final concentration of 30 mM Tris-hydrochloride (pH 8.0), 4 mM magnesium acetate, 0.65 mM dithiothreitol, 0.8 mM concentrations of the three unlabeled triphosphates, and 0.5 mM concentrations of the α-^{32}P-labeled triphosphates (500 μCi/μmol), 7.5 μM [^{3}H]UTP (625 μCi/μmol), or 0.6 mM [^{3}H]ATP (83 μCi/μmol). Samples were incubated at 31°C for the indicated times, and the labeled RNA was purified by sodium dodecyl sulfate (SDS)-phenol extraction or trichloroacetic acid precipitated and then collected on cellulose acetate filters and counted.

RNA purification. Labeled RNA was purified by extraction with phenol-chloroform-isoamyl alcohol (1:1:0.04). The RNA was then precipitated from the aqueous phase by addition of 2 volumes of ethanol.

(i) In vitro RNA. If the labeled product of an in vitro reaction was to be used for hybridization experiments, the nucleocapsid was first removed by centrifugation for 90 min at 38,000 rpm in an SW50.1 rotor before phenol extraction. Free nucleotides and small molecules were removed by Sephadex G-50 (Sigma) chromatography.

(ii) Virion RNA. ^{3}H-labeled virion RNA was purified from preparations of B and T particles obtained by one rate zonal centrifugation step. The 42S and 30S virion RNAs were separated by centrifugation at 16,500 rpm for 16 h in an SW27 rotor, using a 10 to 30% sucrose gradient containing TES buffer (50 mM Tris-hydrochloride [pH 7.6]–2 mM EDTA–0.2% SDS). RNA sedimenting as 42S and 30S, respectively, was pooled and reprecipitated with ethanol. The virion RNA was partially fragmented by incubation at 50°C for 7 min in 50 mM Na_2CO_3 and ethanol precipitated before hybridization.

(iii) mRNA. ^{32}P-labeled viral mRNA was harvested from infected L cells incubated for 5 h in phosphate-free medium 199 containing 80 μCi of [^{32}P]orthophosphate per ml. The monolayers were washed twice with Earle basic salts solution and lysed with basic salt solution containing 1% Triton X-100, 1 mg of heparin per ml, 90 μg of cycloheximide per ml, and 250 μg of spermidine per ml. Contaminating nuclei were removed by low-speed centrifugation, and the supernatant was layered over 1 ml of 1 M sucrose in 50 mM Tris-hydrochloride (pH 7.4)–25 mM KCl–5 mM $MgCl_2$ and centrifuged in the SW50.1 rotor at 41,000 rpm for 2.5 h. RNA was extracted from the resultant polysome pellet and ethanol precipitated, and the poly(A)-containing material was selected by chromatography on oligo(dT)-cellulose columns. The poly(A)-containing RNA was resolved on 10 to 30% sucrose gradients containing TES buffer by centrifugation at 20,000 rpm for 16.5 h in the SW27 rotor. RNA was located by Cerenkov counting and the labeled RNA was recovered from the appropriate fractions by ethanol precipitation.

Acrylamide gels. The 2% acrylamide gels contained 2% acrylamide, 0.1% Bis, and 0.9% agarose, whereas the 20% gels contained 20% acrylamide, 0.075% Bis, and 8 M urea (11). Electrophoresis buffer consisted of 35 mM Tris base, 30 mM NaH_2PO_4, 1 mM disodium EDTA, and 0.2% SDS, pH 7.6. Electrophoresis was at 5 mA/cylindrical gel until the bromophenol blue marker was 1 to 2 cm from the bottom. Gels were fractionated with a Mickle gel slicer, incubated at 50°C in 0.5 ml of Nuclear-Chicago solubilizer diluted with water (9:1), mixed with 10 ml of toleune-based scintillation fluid, and counted on a Beckman scintillation counter.

Oligonucleotide fingerprinting. RNA products synthesized in vitro with [^{3}H]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP, or [α-^{32}P]UTP were purified from polymerase assays, and unincorporated ribonucleoside triphosphates were removed by chromatography on Sephadex G-50. Digestion of labeled product with bacterial alkaline phosphatase (Worthington) before sequence analysis followed published procedures (19). Bacterial alkaline phosphatase was removed from the reaction mix by addition of SDS to 0.5% and extraction with water-saturated phenol. RNA was desalted before digestion with RNase T1 by chromatography on Sephadex G-10.

For RNase T1 digestion, 20 μg of yeast RNA was added to each sample, and the sample was ethanol precipitated. Precipitated RNA was dissolved in 2 μl of a buffer containing 10 mM Tris (pH 7.6), 0.5 mM EDTA, and 8 U of RNase T1 (Calbiochem) and incubated at 37°C for 30 min. ^{32}P-labeled RNase T1-resistant oligonucleotides were resolved by a two-dimensional fingerprinting system utilizing electrophoresis on strips of cellulose acetate (Schleicher and Schuell) at pH 3.5 in the first dimension (24) and homochromatography on polyethyleneimine-cellulose thin layers (Polygram Cel-300 PEI, Machery-Nagel) in the second dimension. Pretreatment of polyethyleneimine sheets and homochromatography were essentially as described by Volckaert et al. (27). The homomixture used for development was

made by hydrolysis of a 30% RNA solution for 5 min at 37°C in 0.75 N KOH, followed by rapid neutralization with concentrated HCl and dilution to yield a solution at a final concentration of 5% RNA and 7 M urea, pH 7.5. Homochromatography was performed at 60°C. Oligonucleotides were located by autoradiography, collected, and then eluted with triethylammonium-carbonate, pH 10 (27). Compositions of the eluted material were determined by subsequent digestion with RNase A (3). Nearest-neighbor analysis of α-^{32}P-nucleoside triphosphate-labeled oligonucleotides was performed by digestion with RNases T1, T2, and A (10).

RESULTS

Purification of LT virions. The VSV_{Ind} virus used in our laboratory, when propagated in BHK cells infected at a high multiplicity of infection, produced T particles that sedimented on sucrose velocity gradients as a single band clearly resolved from the B-particle band. RNA isolated from pure preparations of B and T particles migrated on polyacrylamide gels with estimated positions equivalent to RNA with sedimentation values of 42S and 30S, respectively. Since this T-particle RNA is longer than the RNA found in most of the other T particles, we have called it LT for "long T particle." The LT and B particles could be purified by three sucrose velocity gradient centrifugations. The purity of these preparations was routinely monitored by extracting [^{3}H]uridine-labeled RNA from either the B or LT virions and determining the percentage of label migrating as 42S B- or 30S LT-virion RNA on polyacrylamide gels. A polyacrylamide gel profile demonstrating the purity of a typical LT-particle preparation is shown in Fig. 1. The purification procedure removed virtually all of the B particles from the LT population, although B-particle preparations normally contain 10 to 20% contaminating LT particles.

LT-virion RNA hybridizes to 28S viral mRNA. To determine the sequence composition of the LT RNA, LT-virion RNA was annealed with purified viral mRNA. ^{32}P-labeled viral mRNA purified from cells infected with a T-particle-free stock of VSV was fractionated by sucrose velocity centrifugation. Fractions containing labeled 28S mRNA (which codes for the L protein) and 13–18S mRNA's (which code for the remaining four viral proteins) were collected, and the purity of the mRNA preparations was verified by polyacrylamide gel electrophoresis (Fig. 2). When the ^{3}H-labeled LT-virion RNA was annealed with increasing amounts of 28S or 13–18S mRNA's, only those samples containing 28S mRNA showed increased RNase resistance (Fig. 3). With the addition of the highest level of 28S mRNA, 82% of the ^{3}H-labeled LT-virion RNA was found in RNase-resistant hybrids, whereas 53% of the added 28S mRNA was also hybridized. ^{3}H-labeled LT-virion RNA did not anneal significantly with the 13–18S mRNA fractions, whereas ^{3}H-labeled B-virion RNA became 37% RNase resistant after annealing to the 13–18S mRNA (data not shown). These results show that most, if not all, of the LT RNA sequences are homologous to the region of the B-particle

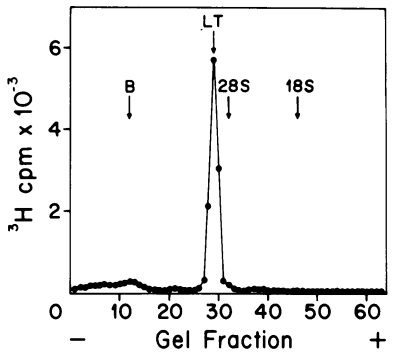

FIG. 1. *Purity of the LT-virion preparation. Virus particles labeled with [^{3}H]uridine were purified by one density and three rate zonal gradient centrifugations. The RNA was extracted from the LT preparation, and its purity was determined by electrophoresis on 2% acrylamide gels. Arrows mark the positions of B- and LT-virion RNAs and 28S and 18S ribosomal RNA markers.*

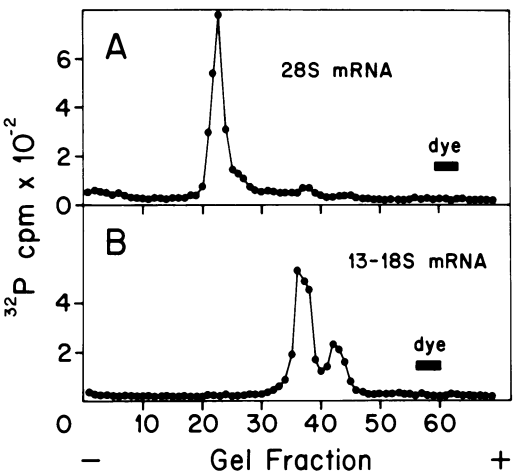

FIG. 2. *Acrylamide gel profiles of purified 28S and 13–18S viral mRNAs. ^{32}P-labeled mRNA was isolated from polysomes and purified by oligo(dT)-cellulose chromatography and sucrose gradient sedimentation. The peak fractions from the sucrose gradients were analyzed on 2% acrylamide gels. (A) 28S mRNA fraction; (B) 13–18S mRNA fraction.*

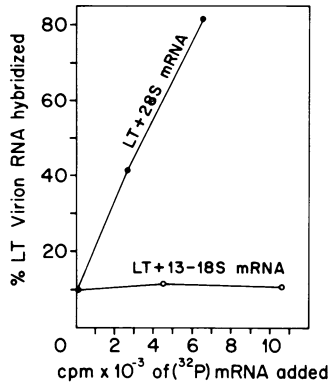

FIG. 3. *Sequence homology of LT-virion RNA with viral mRNA's. Different amounts of ^{32}P-labeled mRNA were mixed with [^{3}H]uridine-labeled LT-virion RNA in 0.4 M NaCl-10 mM Tris-hydrochloride (pH 7.4) in a total volume of 80 μl. Samples were boiled for 30 s and then annealed at 68°C for 2.5 h. Each sample was diluted to 2.1 ml with 0.15 M NaCl-10 mM Tris-hydrochloride (pH 7.4) and divided into four 0.5-ml fractions. Nine units each of RNase A and RNase T1 were added to half of the tubes, and all samples were incubated at 37°C for 30 min and then precipitated with trichloroacetic acid, collected on cellulose acetate filters, and counted. Each tube contained 2,500 cpm of ^{3}H-labeled LT-virion RNA. The percentage of hybridization was calculated as in Table 1, footnote c. LT-virion RNA annealed with 13-18S mRNA* (○) *or 28S mRNA* (●).

genome coding for the 28S mRNA, which means that they represent all or part of the L-protein gene.

LT particles synthesize RNA in vitro. B and LT particles were purified from a single infection and tested in a standard in vitro polymerase assay. LT particles incorporate either [^{3}H]ATP or [^{3}H]UTP into trichloroacetic acid-precipitable material for up to 5 h (Fig. 4). A comparison of the extent of incorporation of [^{3}H]UTP by similar amounts of B and LT particles showed that LT particles synthesized less than 10% as much RNA as B particles (data not shown). The LT product did not contain significant regions of poly(A) since the [^{3}H]ATP-labeled LT product was completely acid soluble after digestion with a mixture of RNase A and RNase T1 (Table 1) and did not bind to oligo(dT) columns in 0.5 M NaCl (data not shown). Characterization of labeled in vitro products on 2% polyacrylamide gels demonstrated that most of the RNA synthesized by B particles was of mRNA size, whereas all of the RNA synthesized by LT particles was much smaller and migrated ahead of the bromophenol blue dye marker (Fig. 5A and B). Analysis of the in vitro products on 20% polyacrylamide gels showed that the majority of the labeled B product failed to enter the gel (Fig. 5C). In contrast, virtually all of the LT product entered the gel and migrated as a sharp band ahead of a 4S marker (Fig. 5D).

LT product hybridizes to LT-virion RNA. To demonstrate that LT-virion RNA was the template for the synthesis of the small RNA species, LT product labeled with [α-^{32}P]CTP or [^{3}H]UTP was annealed with either 42S B-particle or 30S LT-particle virion RNA that had been partially fragmented with dilute alkali. Seventy-three percent of the LT product hybridized

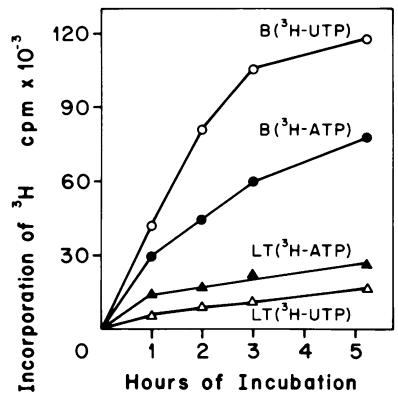

FIG. 4. *Kinetics of in vitro RNA synthesis by purified B and LT virions. [^{3}H]uridine-labeled virions were purified and assayed for polymerase activity as described in the text. Duplicate samples were assayed, and the background at zero time (5,500 to 7,500 cpm) was subtracted. B virions (circles) and LT virions (triangles) were incubated with [^{3}H]UTP (open symbols) or [^{3}H]ATP (closed symbols).*

TABLE 1. *RNase sensitivity of in vitro polymerase products*

Sample[a]	Trichloroacetic acid-insoluble cpm[b]		RNase resistant[c] (%)
	−RNase	+RNase	
LT product ([^{3}H]ATP)	4,772	47	0.98
LT product ([^{3}H]UTP)	1,560	46	2.95
B product ([^{3}H]ATP)	14,870	1,941	13.05
B product ([^{3}H]UTP)	10,585	49	0.46

[a] In vitro products from the 5-h, 15-min reaction shown in Fig. 4 were centrifuged to remove template, phenol extracted, and chromatographed on Sephadex columns to remove low-molecular-weight components.

[b] RNA was annealed for 2 h at 72°C in 100 μl of 0.4 M NaCl-10 mM Tris-hydrochloride (pH 7.4) and then treated with RNase A and RNase T1 as described in the legend to Fig. 3.

[c] Calculated as (trichloroacetic acid-insoluble cpm after RNase/trichloroacetic acid-insoluble cpm before RNase) × 100.

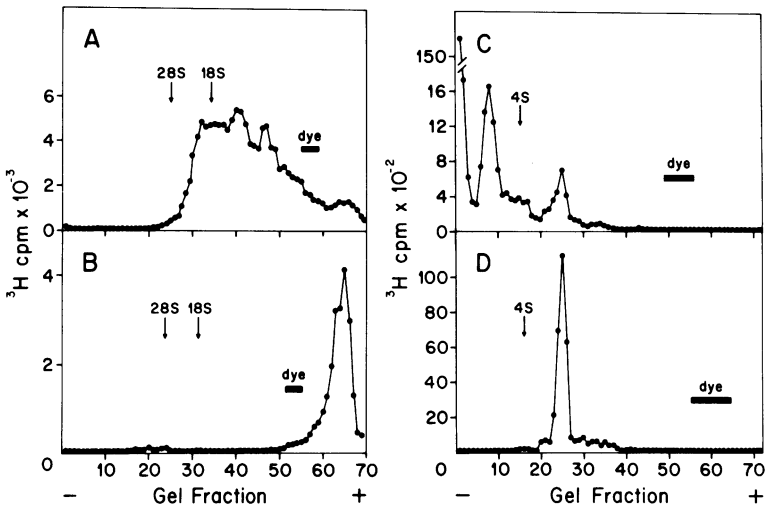

FIG. 5. *Acrylamide gel profiles of RNAs synthesized in vitro by purified B and LT virions. [^{3}H]UTP-labeled products from a 3-h in vitro polymerase reaction were separated from template by centrifugation, phenol extracted, and separated from unincorporated nucleotides and small molecules by Sephadex chromatography before analysis on acrylamide gels. Arrows denote the position of marker RNAs. (A) 2% gel, B-virion product; (B) 2% gel, LT-virion product; (C) 20% gel, B-virion product; (D) 20% gel, LT-virion product.*

to LT-virion RNA at approximately a 3:1 (virion:product) molar ratio of input RNAs (Fig. 6). Annealing of labeled LT product with B-particle RNA yielded little, if any, hybridization, although both B- and LT-virion RNAs had the same specific activity and similar amounts of these RNAs were added to the annealing mixtures. Lack of annealing to B-virion RNA may reflect technical difficulties in the hybridization procedure or could indicate that generation of the LT particle resulted in the acquisition of new or modified sequences. We are currently trying to differentiate between these two possibilities. Although we have not been able to demonstrate the presence of sequences complementary to LT product in B-particle virion RNA, it is clear from our data that LT-virion RNA is the template for the synthesis of LT product.

Oligonucleotide fingerprint of LT product. To directly estimate the complexity of the small LT RNA synthesized by LT particles, LT product was synthesized in vitro with labeled [α-^{32}P]GTP and subjected to RNase T1 digestion. The resulting T1 oligonucleotides, containing at least one labeled P residue per oligonucleotide, were separated by electrophoresis on cellulose acetate followed by homochromatography.

Figure 7 shows an oligonucleotide fingerprint of LT product synthesized using [α-^{32}P]GTP and digested with RNase T1. Six major oligonucleotides can be identified, suggesting that the LT product is a relatively homogeneous species

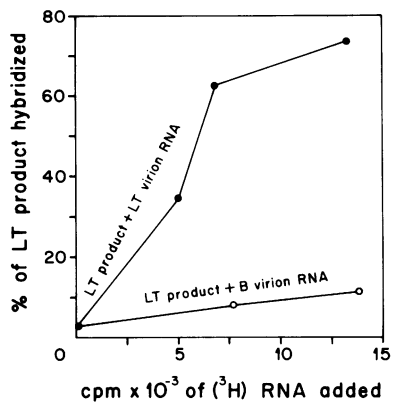

FIG. 6. *Hybridization of LT in vitro product to LT-virion RNA. [^{32}P]CTP-labeled LT product was mixed with increasing amounts of [^{3}H]uridine-labeled purified B- or LT-virion RNA in a volume of 55 μl of 0.3 M NaCl-0.3 M sodium citrate, pH 7.0. Samples were boiled for 30 s and incubated at 70°C for 17.5 h. Each sample was diluted to 2.1 ml with 0.3 M NaCl-10 mM Tris-hydrochloride (pH 7.4), and 0.5-ml fractions were analyzed as in Fig. 3. Each 0.5-ml fraction contained 1,600 cpm of ^{32}P-labeled product. The ^{3}H-labeled virion RNAs contained approximately 13,500 cpm/μg. LT product hybridized to B-virion RNA (○) or LT-virion RNA (●).*

with low complexity. Oligonucleotide fingerprint analysis of [α-^{32}P]GTP-labeled LT product digested first with bacterial alkaline phosphatase and subsequently with RNase T1 revealed

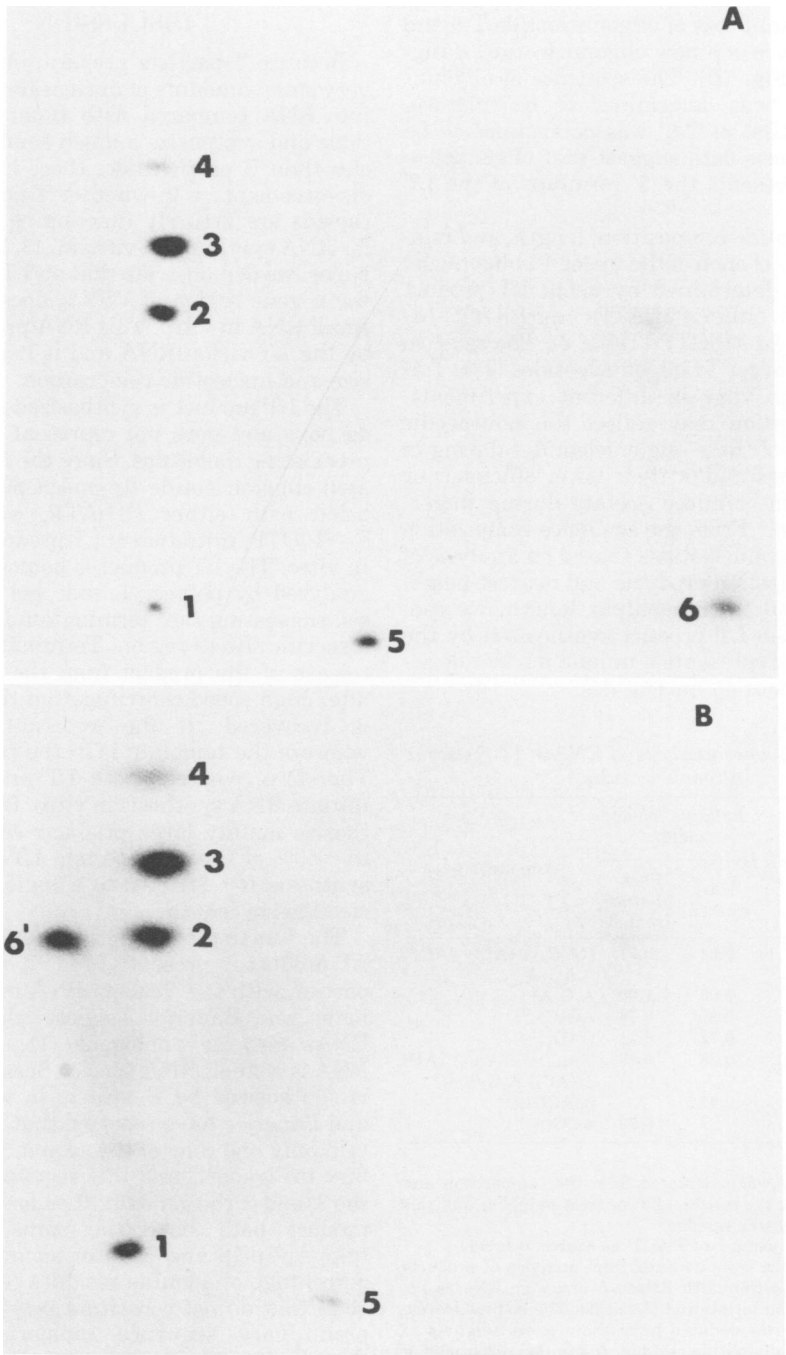

FIG. 7. *Two-dimensional oligonucleotide fingerprints of [α-^{32}P]GTP-labeled RNA synthesized in vitro by LT particles. ^{32}P-labeled product from a single preparation was divided into two equal portions. One portion (A) was digested with RNase T1 and fingerprinted as described in Materials and Methods. The second portion (B) was first treated with bacterial alkaline phosphatase and then RNase T1 as described in Materials and Methods. The direction of electrophoresis in the first dimension on strips of cellulose acetate in 5 M urea, pH 3.5, is from left to right. Homochromatography in the second dimension on polyethyleneimine-cellulose thin layers at pH 7.5 is from bottom to top.*

the concomitant loss of oligonucleotide T-6 and the appearance of a new oligonucleotide, designated T-6′ (Fig. 7B). The sequence of oligonucleotide T-6 was determined to be (p)ppApCpGp, and that of T-6′ was determined to be ApCpGp. These data suggest that oligonucleotide T-6 represents the 5′ terminus of the LT product.

The nucleotide composition, length, and relative amounts of each of the major T1 oligonucleotides were determined by using LT product labeled with either [^{3}H]ATP, [α-^{32}P]CTP, [α-^{32}P]UTP, or [α-^{32}P]GTP (Table 2). The relative yield of the larger T1 oligonucleotides (T-1, T-5) was found to vary in different experiments. This observation may reflect the nonspecific degradation of these oligonucleotides during in vitro synthesis and/or their lower efficiency or transfer from cellulose acetate during fingerprint analysis. From the sequence composition of the T1 oligonucleotides (based on analysis of RNase A digestion products and nearest-neighbor data) and their apparent length, we conclude that the LT product synthesized by the LT particles represents a unique nucleotide sequence of about 45 nucleotides.

TABLE 2. *Sequence analysis of RNAse T1-resistant oligonucleotides*

Oligonucleotide no.[a]	Complexity[b]	Relative molar yield[c]		Composition[d]
		Untreated	Phosphatase treated	
T-1	13	0.64	0.51	[(A_4C_2) (AC) (AC_2)] AG(A)
T-2	3	1.00	1.00	A_2G(A)
T-3	2	0.82	0.76	AG(G)
T-4	1	0.72	0.71	G (G,U)[e]
T-5	20–21	0.38	0.20	[(A_{5-6} U) (A_5C_2) (AU) (AC)] A_2G(A)
T-6	3	0.41		ppACG(A)[f]
T-6′	3		0.79	ACG(A)

[a] See Fig. 7.

[b] Complexity was calculated from the composition and does not include the residue that nearest-neighbor analysis labels the guanosine residue.

[c] A_2G = 1.0, using [α-^{32}P]GTP as source of label.

[d] Compositions were deduced from analysis of products obtained by digestion with RNase A and with RNases T1, T2, and A (see Materials and Methods). The last nucleotide in parentheses was deduced to be the nearest neighbor to the 3′-terminal guanosine residue. A detailed discussion of the sequence analysis will be presented elsewhere (manuscript in preparation).

[e] Nearest neighbor labeled with both [α-^{32}P]GMP and [α-^{32}P]UMP.

[f] Oligonucleotide 6, labeled with [^{3}H]ATP and [α-^{32}P]CTP, released a product containing both ^{32}P and ^{3}H labels and migrated on 3MM paper (pH 3.5) as ppAp after digestion with RNases T1, T2, and A.

DISCUSSION

Because T-particle preparations incorporate very small amounts of nucleoside triphosphates into RNA compared with B-particle preparations and synthesize a much smaller RNA species than B particles do, there has been some disagreement as to whether T-particle nucleocapsids are actually functioning as templates for RNA synthesis in vitro (8, 13, 20, 23). In this paper, we demonstrate that an LT particle from the *L*-gene region of VSV_{Ind} does synthesize a small RNA in vitro. This RNA product is coded by the LT-virion RNA and is homogeneous in size and nucleotide composition.

The LT product is synthesized in its entirety de novo and does not represent elongation of preexisting molecules. Since the 5′-phosphorylated oligonucleotide ([p]ppApCpGp) can be labeled with either [^{3}H]ATP, [α-^{32}P]CTP, or [α-^{32}P]GTP, initiation of LT product must occur in vitro. The LT product is homogeneous when analyzed by polyacrylamide gel electrophoresis, suggesting that termination is occurring at a specific site or region. Termination results in release of the product from the template, for after high-speed centrifugation the LT product is recovered in the supernatant fraction, whereas the template is in the pellet fraction. Therefore, whereas both LT and B particles initiate RNA synthesis in vitro, B particles synthesize mainly large products complementary to ~50% of the genome and LT-particle RNA synthesis is restricted to a region only 45 nucleotides in length.

The function or biological significance of the LT product is presently undefined, but a comparison with the "leader" RNA reported by Colonno and Banerjee suggests that these two RNAs may be analogous (11, 12). "Leader" RNA is a small RNA species (less than 4S) that is synthesized by B virions in vitro. Colonno and Banerjee have reported that B virions contain only one copy of the sequence complementary to "leader," and this sequence constitutes the 3′ end of the genome. "Leader" RNA and LT product both have the same 5′ sequence (p)ppApCpGp and contain an unusually high percentage of adenine residues (approximately 50%) that do not constitute poly(A). However, preliminary sequence comparisons indicate that the primary sequences of "leader" and LT product are not identical (A. K. Banerjee, personal communication). This may reflect differences in the virus stocks used or the synthesis of LT product from a different region of the viral genome. At this time we have been unable to purify enough "leader" from our preparations

of B-virion transcription products to allow a direct comparison.

Are the "leader" and LT-product RNAs produced by the same mechanism? Colonno and Banerjee have postulated that "leader" RNA represents a by-product of mRNA processing (11). That is, they suggest that during transcription, the RNA polymerase initiates at the ultimate base of the 3′ end of the B genome and synthesizes "leader" as the 5′ terminus of a longer RNA, which is subsequently cleaved by a nuclease to generate "leader" and the individual mRNA's. During replication, processing would not occur and genome-length RNA would be synthesized. However, to date no one has reported the detection of either such a nuclease or a precursor molecule. On the other hand, since the 45-nucleotide-long LT product appears homogeneous and no large quantities of other products have been detected, we must consider the possibility that its discrete size reflects termination of synthesis rather than cleavage from a larger molecule.

It may be that LT product synthesis is a result of replication starts rather than transcription. In vivo replication of VSV nucleic acid requires concomitant protein synthesis, for production of genome-length RNA quickly stops when cycloheximide is added to infected cells (18, 22). Since a characteristic feature of VSV T particles is their ability to replicate efficiently in cells when co-infecting B particles are present, we suggest that the in vitro synthesis of the LT product, as well as the "leader" RNA, represents replication that is aborted because one or more factors normally provided in vivo by newly synthesized proteins are absent. Further experiments should resolve these alternatives, and the information obtained should greatly increase our understanding of both VSV replication and T-particle generation.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants AI-11722 from the National Institute of Allergy and Infectious Diseases (S.U.E.) and CA 16553 from the National Cancer Institute (J.T.P.) and by grant VC-186 from the American Cancer Society (J.T.P.). S.U.E. is the recipient of Public Health Service Research Career Development Award AI-00013 from the National Institute of Allergy and Infectious Diseases.

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., D. P. Rhodes, and A. K. Banerjee.** 1975. The 5′-terminal structure of the methylated mRNA synthesized *in vitro* by vesicular stomatitis virus. Cell 5:51–58.
3. **Adams, J. M., P. G. N. Jeppesen, F. Sanger, and B. G. Barrell.** 1969. Nucleotide sequence from the coat protein cistron of bacteriophage R17. Nature (London) 223:1009–1014.
4. **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.
5. **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.
6. **Banerjee, A. K., G. Abraham, and R. J. Colonno.** 1977. Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. 34:1–8.
7. **Banerjee, A. K., and D. P. Rhodes.** 1973. *In vitro* synthesis of RNA that contains polyadenylate by virion associated RNA polymerase of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. 70:3566–3570.
8. **Bishop, D. H. L., and P. Roy.** 1971. Kinetics of RNA synthesis by vesicular stomatitis virus particles. J. Mol. Biol. 57:513–527.
9. **Both, G. W., S. A. Moyer, and A. K. Banerjee.** 1975. Translation and identification of the mRNA species synthesized *in vitro* by the virion-associated RNA polymerase of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. 72:274–278.
10. **Cashion, L. M., R. H. Joho, M. A. Planitz, M. A. Billeter, and C. Weissmann.** 1976. Initiation sites of Rous sarcoma virus RNA-directed DNA synthesis *in vitro*. Nature (London) 262:186–190.
11. **Colonno, R. J., and A. K. Banerjee.** 1976. A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription *in vitro*. Cell 8:197–204.
12. **Colonno, R. J., and A. K. Banerjee.** 1977. Mapping and initiation studies on the leader RNA of vesicular stomatitis virus. Virology 77:260–268.
13. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. 10:297–309.
14. **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.
15. **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.
16. **Galet, H., D. Hallet, and L. Prevec.** 1976. Analysis of in vitro transcription products of intracellular vesicular stomatitis virus RNA polymerase. J. Virol. 19:467–474.
17. **Huang, A. S.** 1973. Defective interfering viruses. Annu. Rev. Microbiol. 27:101–117.
18. **Huang, A. S., and E. K. Manders.** 1972. Ribonucleic acid synthesis of vesicular stomatitis virus. IV. Transcription by standard virus in the presence of defective interfering particles. J. Virol. 9:909–916.
19. **Min Jou, W., and W. Fiers.** 1969. Studies on bacteriophage MS2. J. Mol. Biol. 40:187–201.
20. **Mori, H., and A. F. Howatson.** 1973. *In vitro* transcriptase activity of vesicular stomatitis virus B and T particles: analysis of product. Intervirology 1:168–175.
21. **Moyer, S. A., and A. K. Banerjee.** 1975. Messenger RNA species synthesized *in vitro* by the virion-associated RNA polymerase of vesicular stomatitis yirus. Cell 4:37–43.
22. **Perlman, S. M., and A. S. Huang.** 1973. RNA synthesis of vesicular stomatitis virus. V. Interactions between transcription and replication. J. Virol. 12:1395–1400.
23. **Reichmann, M. E., L. P. Villarreal, D. Kohne, J. Lesnaw, and J. J. Holland.** 1974. RNA polymerase activity and poly(A) synthesizing activity in defective T

particles of vesicular stomatitis virus. Virology 58:240–249.
24. **Sanger, F., G. G. Brownlee, and B. G. Barrell.** 1965. A two-dimensional fractionation procedure for radioactive nucleotides. J. Mol. Biol. 13:373–398.
25. **Schnitzlein, W. M., and M. E. Reichmann.** 1976. The size and the cistronic origin of defective vesicular stomatitis virus particle RNAs in relation to homotypic and heterotypic interference. J. Mol. Biol. 101:307–325.
26. **Villarreal, L. P., and J. J. Holland.** 1973. Synthesis of poly(A) *in vitro* by purified virions of vesicular stomatitis virus. Nature (London) New Biol. 246:17–19.
27. **Volckaert, G., W. Min Jou, and W. Fiers.** 1976. Analysis of ^{32}P labelled bacteriophage MS2 RNA by a mini-fingerprinting procedure. Anal. Biochem. 72:433–446.
28. **Wagner, R. R.** 1975. Reproduction of rhabdoviruses, p. 1–93. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 4. Plenum Press, New York.