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L Protein Requirement for In Vitro RNA Synthesis by Vesicular Stomatitis Virus

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The endogenous transcriptase present in purified vesicular stomatitis (VS) virions was solubilized with a Triton X-100 high-salt solution. The polymerase activity was purified on glycerol gradients and by phosphocellulose column chromatography; the viral proteins present in the active enzyme fractions were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It was demonstrated that L protein, but not NS protein, was required for in vitro RNA synthesis on the VS viral nucleocapsid template. Solubilized L protein rebinds to the ribonucleoprotein template when the transcription complex is reconstituted, and the RNA synthesized in vitro by purified L protein hybridizes to virion RNA. Cyanogen bromide peptide fingerprints indicate that the large L protein is a unique polypeptide chain. It is concluded that the L protein functions as the transcriptase, and the nucleocapsid NS protein is not essential for in vitro RNA synthesis.

Vesicular stomatitis (VS) virions contain an endogenous RNA-dependent RNA polymerase that functions as a transcriptase both in vivo and in vitro (1, 2, 4, 10). The identification of temperature-sensitive mutants that are defective in transcription indicates that viral proteins are required for this process (6, 13, 17). Virions disrupted with Triton X-100 and NaCl can be separated by centrifugation into supernatant fluid and pellet fractions that are deficient in transcriptase activity (7). Transcriptase activity is reconstituted upon recombination of these two fractions. Each fraction contains a heat-labile component, suggesting that at least two proteins are required for RNA synthesis. Because the pellet fraction contains all of the nucleocapsid (N) protein but little, if any, of the other viral proteins and because deproteinized RNA does not serve as template (3, 7), N protein is believed to be a necessary component of the transcription complex. The supernatant fluid fraction contains the remaining four viral proteins: L (large), G (glycoprotein), M (membrane), and NS (originally thought to be nonstructural). Three different laboratories have demonstrated that the envelope proteins, G and M, are not required for transcription (5, 7, 18). Therefore, either L or NS or both proteins could be the supernatant fluid factor required for transcription.

In this report, we propose that the large viral protein L is a single polypeptide chain and is required for in vitro RNA synthesis by VS virus (VSV).

MATERIALS AND METHODS

Chemicals and radiochemicals. Unlabeled nucleoside triphosphates and dithiothreitol (DTT) were obtained from Calbiochem, La Jolla, Calif. Bio-Solv (BBS-2 and BBS-3) was purchased from Beckman Instruments Inc., Silver Spring, Md. RNase A (4,600 U/mg) was from Worthington Biochemical Corp., Freehold, N.J., whereas Triton X-100, ribonuclease T_1 (367,400 U/mg), and Trizma Base were from Sigma Chemical Co., St. Louis, Mo. ^{3}H-UTP (15 Ci/mmol), ^{3}H-leucine (51 Ci/mmol), [^{3}H]tyrosine (52 Ci/mmol), ^{3}H-methionine (general label, 100 to 700 mCi/mmol) and ^{14}C-methionine (carboxyl label, 5 to 15 mCi/mmol) were purchased from Schwarz/Mann, Orangeburg, N.Y.; ^{14}C-protein hydrolysate (57 mCi/matom of carbon) was purchased from Amersham/Searle, Arlington Heights, Ill. Cyanogen bromide (CNBr) was from Matheson, Coleman and Bell, Norwood, Ohio, whereas the formic acid was from the Mallinckrodt Chemical Works, Jersey City, N.J. Nitrocellulose filters (0.45-μm pore) were purchased from Matheson-Higgins Co., Woburn, Mass. Glycerol, sodium chloride, and other chemicals were from Fisher Scientific Co., Fair Lawn, N.J.

Solutions. Triton-high-salt solubilizer consisted of the following: 1 ml of glycerol, 1 ml of DTT (1 mg/ml of H_2O), 1 ml of Triton X-100 (20% stock in H_2O), 444 mg of NaCl, and 2.35 ml of reticulocyte standard buffer (RSB). RSB consisted of 0.01 M KCl, 0.0015 M $MgSO_4$, and 0.01 M Tris-hydrochloride (pH 7.4). To obtain polymerase reaction mixture, mix 20 μliters of ^{3}H-UTP (15 Ci/mmol) and 0.1 ml of unlabeled UTP

(0.12 mg/ml), lyophilize, and then add 1.0 ml of prereaction solution (1.4 mM ATP, GTP, and CTP, 8 mM magnesium acetate, 0.65 mM DTT, and 0.5 mM Tris-hydrochloride, pH 7.4). Diluent solution consisted of 1 volume of Triton-high-salt solubilizer and 1.5 volumes of RSB or Tris-hydrochloride. Glycerol column wash consisted of the following: 75 ml of glycerol, 3 ml of Triton X-100 (20% stock), 9 mg of DTT, and enough 0.05 M Tris-hydrochloride (pH 7.4) to adjust the final volume to 300 ml. Swank and Munkres tray buffer consisted of 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris and contained 0.1% sodium dodecyl sulfate (SDS).

Viruses and cells. Cultivation of the Indiana strain of VS virus has been described previously (21, 22). VS virions were grown on L cells in Eagle basal medium diluted 1:50 with Earle balanced salt solution. Radioactive isotopes were added along with the medium after the initial adsorption of virus to cells. Virus was harvested after 18 to 20 h of growth. The media containing the released virions were clarified by centrifugation at 1,000 × *g* for 10 min to remove cellular debris. The media were then centrifuged for 90 min at 80,000 × *g* in an SW27 rotor to pellet the virus through a 50% glycerol pad made by diluting one volume of glycerol with one volume of Earle balanced salt solution. The pellets were suspended in Earle balanced salt solution, sonically treated to disperse aggregates, and layered on top of a preformed, linear 0 to 40% sucrose gradient. This gradient removes residual cellular membrane fragments from the virions and allows separation of B and T particles. After centrifugation in an SW25.1 rotor for 90 min at 35,000 × *g*, the visible B band was removed by side puncture of the tube, and the virions were repelleted in an SW50L rotor at 80,000 × *g* for 60 min. The purified virions were suspended in RSB or 0.01 M Tris (pH 7.4) to which DTT (0.05 mg/ml) was added. Virions to be used for the polymerase assay were not frozen, but were stored at 4 C and used within 2 days.

Solubilization and reassociation of virion proteins. Solubilization was performed essentially as described previously (7). Virus in either RSB or 0.01 M Tris-hydrochloride (pH 7.4) was mixed with an equal volume of 2X-concentrated Triton-high-salt solubilizer at a final concentration of about 0.1 to 0.2 mg of protein per ml to prepare VS nucleocapsid template or 0.4 to 0.8 mg of protein per ml to prepare soluble enzyme. The release of L and NS proteins from the ribonucleoprotein complex (RNP) is very dependent on the NaCl and protein concentrations and is efficient only if the protein concentration is low and the ionic strength is high. The sample was gently mixed and incubated at 31 C for 1 h without stirring. As long as the NaCl concentration is kept at 0.72 M and the amount of viral protein is kept low, the L, NS, G, and M proteins can be separated from the nucleocapsid by centrifugation at 125,000 × *g*, as previously described; if the NaCl concentration is lowered to 0.288 M, some L protein will precipitate although the remaining L and virtually all of the NS protein will reassociate with the RNP (Fig. 1). Therefore, to reconstitute an active transcription system, appropriate viral components from the supernatant were mixed with the RNP at an NaCl concentration of 0.288 M.

Transcriptase assay. This assay system has been previously described (7), but the enzyme is so labile and the conditions for maximal activity are so exacting that a detailed description seems warranted. Template was mixed with various preparations of supernatant proteins to give a solution equivalent to diluent solution; because diluent solution contains 0.288 M NaCl, reassociation of polymerase and template occurs. The polymerase reaction has an optimum NaCl concentration of approximately 0.15 M (unpublished data), so the samples were diluted with one volume of polymerase reaction mixture to yield a final NaCl value of 0.144 M. Samples (0.1 ml) were distributed to test tubes with Corning 1-ml disposable glass pipettes, capped, and placed in a 31 C water bath without shaking to allow RNA synthesis. Tubes were withdrawn from the water bath (usually at 1-h intervals); 0.1 ml of yeast carrier RNA (2 mg/ml) and 0.6 ml of 0.067 M sodium pyrophosphate were added; and the samples were then frozen until precipitated (1). An unincubated sample served as a 0 time control and was treated similarly. Duplicate tubes were processed for each time point. To quantitate RNA synthesis, samples were mixed with 0.5 ml of 25% trichloroacetic acid, kept on ice for 20 min, and filtered through nitrocellulose filters that were then washed five times with 5% trichloroacetic acid. The filters containing the precipitated RNA and proteins were placed in scintillation vials and incubated at room temperature for at least 30 min with 0.3 ml of 0.3 N NaOH to solubilize the precipitate. Toluene-based scintillation fluid (7.5 ml) containing 7% BBS-3 and 3% BBS-2 was added to each vial. After the filters had cleared, samples were counted in a liquid scintillation spectrometer. Unincubated samples generally had a background of ^{3}H-UTP contamination of 100 to 200 counts/min. Template preparations incubated with diluent solution in the absence of added supernatant fraction served to quantitate residual activity due to incomplete removal of polymerase from the RNP; if large amounts of template such as those required to assay column fractions were prepared, residual activity was usually present. Small amounts of template can be prepared, however, that show no detectable RNA synthesis. The supernatant fraction by itself never stimulated ^{3}H-UTP incorporation.

SDS acrylamide gel electrophoresis. Protein samples to be examined were mixed with 50 μg of hemoglobin carrier and one volume of 20% trichloroacetic acid to precipitate the proteins and remove salts. The precipitate was pelleted by centrifugation at 2,000 × *g* for 10 min at 4 C, and the pellet was washed once with 10% trichloroacetic acid and twice with acetone. The precipitate was redissolved in a solution of 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 8 M urea, 1% SDS, and 1% mercaptoethanol and was boiled for 2 min (11). Electrophoresis was on 7.5% SDS-polyacrylamide gels that were processed as previously described (22).

Phosphocellulose column. Whatman P11 cellulose phosphate (W. and R. Balston, Ltd., Maidstone, Kent, England) was washed sequentially with 0.5 M NaOH, H_2O, 0.5 M HCl, and H_2O and equilibrated with 0.5 M Tris-hydrochloride (pH 7.4) containing

0.001 M disodium ethylenediaminetetraacetate. Columns made from 6-ml plastic syringes were prepared by adding 2 to 3 ml of phosphocellulose and then by washing the column with at least 25 ml of glycerol column wash prior to loading the sample. Columns were eluted at 4 C, and a new column was prepared each time. The flow rate was 0.5 to 1.0 ml/5 min, and protein was eluted by raising the NaCl concentration in the glycerol column wash to 0.5 M, and then to 1.0 M.

Glycerol gradient. A 5-ml linear 10 to 35% glycerol gradient was formed over 0.5 ml of 80% glycerol by using a Buchler gradient maker. The 80% glycerol solution was made by mixing 1 ml of DTT (1 mg/ml), 0.1 ml of a 20% stock of Triton X-100, 417 mg of NaCl, and 1 ml of 0.5 M Tris-hydrochloride (pH 7.4) until the NaCl had dissolved. An 8-ml portion of glycerol was then added. The 10 and 35% glycerol solutions were made by mixing 1.0 ml of DTT (1 mg/ml), 0.1 ml of 20% Triton X-100, 417 mg of NaCl, 1 or 3.5 ml of glycerol, and by diluting to a final volume of 10 ml with RSB. The gradient was prepared and centrifuged at 4 C.

RNA hybridization. A transcription complex reconstituted by addition of template- and phosphocellulose-purified L protein was incubated at 31 C in the standard polymerase assay system. The sample was then centrifuged for 90 min at 125,000 × *g* to remove the unlabeled virion RNA in the RNP from the released ^{3}H-labeled in vitro product which remained in the supernatant fraction. The supernatant fraction was made 1% with respect to SDS, mixed with 50 μg of carrier yeast RNA, and extracted with phenol saturated with 0.01 M acetate buffer (pH 5.1) containing 0.1 M NaCl. The aqueous phase was removed, and the RNA was purified from it by ethanol precipitation. Unlabeled virion RNA was phenol extracted from virions in 0.01 M Tris-hydrochloride containing 0.1 M NaCl and 1% SDS and was also ethanol precipitated. The ethanol precipitates were dissolved in 0.01 M Tris-hydrochloride (pH 7.4) containing 0.4 M NaCl. Because no yeast RNA carrier was added to the virion RNA preparation, the amount of RNA was calculated from the optical density at 260 nm, assuming that 40 μg of RNA per ml has an optical density of 1.0. The in vitro product (25 μliters) was mixed with 50 μliters of the Tris-sodium chloride solution containing either 2 μg of viral RNA, 2 μg of yeast RNA, or no added RNA (4). Samples were boiled for 30 s and then were quickly cooled on ice or incubated at 60 C for 105 min. Some samples were then treated with 3,674 U of T_1 RNase and 230 U of RNase A at 22 C for 45 min (1). All samples were next mixed with yeast carrier RNA and pyrophosphate, trichloroacetic acid precipitated, collected on filters, and counted as described for the polymerase assay.

Cyanogen bromide cleavage of proteins. Viral proteins were labeled with ^{14}C-methionine or ^{3}H-methionine, and the viruses were purified by differential centrifugation and sucrose-gradient banding. The two purified virus preparations were treated with SDS, urea, and mercaptoethanol and boiled, and each set was electrophoresed on six 7.5% gels as described in a previous section; sequanal grade SDS (Pierce Chemical Co., Rockford, Ill.) was used throughout this procedure. A reference gel was sliced and counted to locate the protein bands. Corresponding slices were removed from the remaining gels, and the individual bands were pooled with like fractions from each gel. To extract the proteins, the gel slices were crushed by forcing them through a 2-ml syringe without a needle; the fragments were then washed with 0.01 M sodium phosphate buffer (pH 7.2) containing 0.05% SDS and 0.1% mercaptoethanol. The liquid was collected with a Pasteur pipette, and a portion was rerun on 7.5% SDS-acrylamide gels to monitor the homogeneity of the purified protein. A ^{14}C-labeled protein was mixed with a ^{3}H-labeled protein and 500 μg of bovine serum albumin as carrier protein, and the samples were lyophilized. The lyophilized powder was dissolved in 1 ml of 70% formic acid containing 4 mg of CNBr; the tube was wrapped in tin foil and incubated at room temperature for 48 h (15). This reaction mixture was then lyophilized, and the proteins were redissolved in water and relyophilized twice more. The final lyophilized powder was dissolved in 200 μliters of tray buffer diluted 1:10 with water and containing 1% SDS, 8 M urea, and 1% mercaptoethanol (16). The solution was boiled for 2 min, and any undissolved residue was removed by centrifugation. The sample was layered onto a 12.5% polyacrylamide gel, as described by Swank and Munkres, and electrophoresed for 17 h at 16 mA/6 tubes (16). To count radioactivity in the highly cross-linked gels, slices were incubated at 37 C overnight in 0.25 ml of 30% alkaline hydrogen peroxide (9), mixed with 1.2 ml of Nuclear-Chicago solubilizer (NCS) that was diluted to nine parts of NCS to one part of H_2O, mixed with 10 ml of toluene-based scintillation fluid, and left at room temperature until the gel slices cleared. The vials were counted in a scintillation spectrometer.

RESULTS

Purified virions were disrupted with Triton-sodium chloride solubilizer and partitioned by centrifugation into a supernatant fraction containing L, G, NS, and M proteins and a pellet fraction of RNP containing the virion RNA complexed with N protein. In vitro transcription requires heat-labile factors from both the soluble and particulate fractions (7); therefore, we have assumed that the soluble fraction provides the actual transcription enzyme, whereas the RNP fraction functions as the template for transcription. Because the putative transcriptase was solubilized by this procedure, we have attempted to purify the enzyme by using standard procedures. The transcriptase activity in the supernatant fraction is stable to dialysis and can be recovered after various manipulations if care is taken not to denature the proteins. Precautions taken to increase the yield of active enzyme include the presence of glycerol, DTT, and Triton X-100 in all solutions. In addition, protein carrier in the form of human hemoglobin was usually added to aid recovery of a small amount of viral proteins and

to help stabilize the enzyme. Fractions from columns or gradients were collected by lowering the effluent tubing directly to the bottom of a test tube containing a solution of hemoglobin and by manually changing tubes because passage of the proteins through a drop counting fraction collector may irreversibly inactivate the enzyme. Corning 1-ml glass pipettes were used for all transferals of the protein solutions because use of microcaps or capillary pipettes appeared to destroy or decrease the transcriptase activity.

Solubilized L and NS proteins rebind to RNP. Because the transcriptase system is reconstituted by mixing inactive particulate and solubilized proteins, solubilized enzyme must rebind to the RNP. Therefore, any protein that is implicated in the transcription process must, as a minimum requirement, associate with the RNP template concomitant with reconstitution of in vitro transcription. G and M proteins are required neither for transcription nor for infectivity (5, 7, 18). L and NS proteins have both been identified in transcriptive complexes and both are present in the soluble fraction, so either of these two proteins could potentially be the transcriptase. The following experiment was performed to determine if L or NS had the capacity to rebind to the RNP template during reinitiation of transcription. Virions labeled with ^{14}C-amino acids were gently mixed with 5.0 ml of Triton-high-salt solubilizer to yield a dilute suspension of virus (~0.1 to 0.2 mg of protein/ml) and to insure maximal separation of RNP and soluble proteins; a comparable quantity of ^{3}H-amino acid-labeled virions was mixed with 1.2 ml of the same Triton-high-salt solubilizer to provide a more concentrated preparation suitable for use as the polymerase fraction. These virus preparations were incubated in the presence of the high-salt solubilizer at 31 C for 1 h to disrupt the virus and then were centrifuged at 125,000 $\times$ g for 90 min at 4 C in an SW50L rotor to yield pellet and supernatant fractions, neither of which contained significant transcriptase activity. The ^{14}C-amino acid-labeled pellet was suspended in 1.5 ml of RSB and mixed with 1.0 ml of undiluted ^{3}H-amino acid-labeled supernatant fluid and 2.5 ml of a UTP deficient reaction solution containing magnesium acetate and the other three nucleoside triphosphates: ATP, GTP, and CTP. These dilutions reduced the NaCl to 0.144 M, the concentration present during transcription reaction conditions. This mixture was incubated at 31 C for 30 min to allow reconstitution; no transcription of trichloroacetic acid-precipitable material occurred because UTP was omitted. Next, the ^{14}C-RNP and any ^{3}H-labeled soluble proteins that may have bound to the RNP were separated from unbound soluble proteins by centrifugation at 125,000 $\times$ g for 90 min through a pad of the reconstitution solution containing 20% glycerol. The resulting pellet was suspended in 0.15 ml of the reconstitution mixture containing 100 μg of hemoglobin, sonically treated, and layered onto a 5.0-ml preformed 5 to 40% linear sucrose gradient made with the UTP deficient reconstitution mixture. After centrifugation at 53,000 $\times$ g for 2 h in an SW50L rotor, fractions were collected manually and assayed for reconstituted transcriptase activity. Transcription was initiated by adding 100 μliters of each fraction to a tube containing ^{3}H-UTP and by incubating the tubes at 31 C for 2.5 h. An unincubated sample served to delineate the protein concentration along the gradient. To determine if any unbound polymerase were present, a duplicate assay was performed in which both UTP and additional template lacking L and NS were added to each gradient fraction prior to incubation. Samples were trichloroacetic acid precipitated and counted according to the procedure described in Materials and Methods.

The results of the transcriptase assay after sucrose gradient centrifugation of reconstituted enzyme and template are shown in Fig. 1. ^{14}C-labeled template and ^{3}H-labeled supernatant proteins cosedimented as a band that contained reconstituted transcription complex as measured by the ability of fractions 6, 7, and 8 to incorporate ^{3}H-UTP into trichloroacetic acid-insoluble material; this band therefore constituted a stable complex that contained components from both the original RNP template and supernatant enzyme fractions.

Both ^{3}H- and ^{14}C-proteins were also found in the gradient pellet, but here the ratio of ^{3}H to ^{14}C was much higher, and the polymerization of ^{3}H-UTP was relatively lower. Addition of new template resulted in a threefold stimulation of ^{3}H-UTP incorporation in fractions 6 through 8, but did not change the amount of UTP incorporated by the pellet fraction (data not shown). This result suggests that the reconstituted particle contains an excess of potentially active transcriptase. In this regard, it is interesting that addition of template to complete virions in a standard transcriptase reaction mixture causes a threefold enhancement of RNA synthesis, whereas addition of the polymerase-containing supernatant fraction to these complete virions has no effect, indicating that virions may contain more transcriptase than they can effectively use (S. U. Emerson, unpublished data).

The proteins donated to the reconstituted

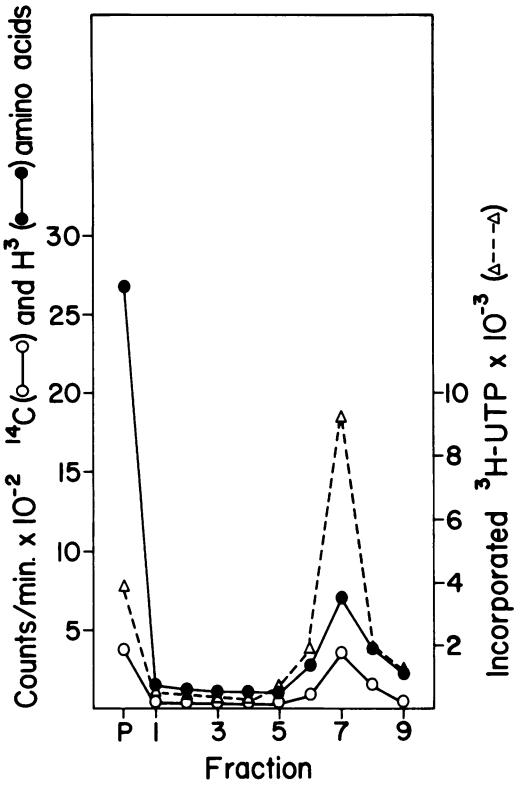

FIG. 1. *Sucrose gradient centrifugation of a reconstituted VS virion transcription complex. After dissociation by Triton-high-salt solubilizer and centrifugation, the ^{14}C-labeled template fraction and ^{3}H-labeled supernatant proteins were mixed, and the resulting complex was pelleted. The pelleted RNP was then centrifuged for 2 h at 53,000 × g on a 5 to 40% sucrose gradient. Fractions of 0.5 ml each were collected, and 50-μliter portions were counted to determine the distribution of ^{14}C- (○) and ^{3}H- (●) labeled protein. Part of each fraction was then incubated with ^{3}H-UTP and the components required for in vitro RNA synthesis to locate the transcription complex. The amount of ^{3}HUTP incorporated into trichloroacetic acid-precipitable material during 2.5 h is plotted (Δ). The supernatant fluid, when assayed before reconstitution with template, incorporated 389 counts/min of ^{3}H-UTP in 2.5 h (background level), whereas the template alone incorporated 538 counts/min during the same time period. P = pelleted, aggregated protein.*

RNP templates by the supernatant fraction were labeled with ^{3}H and, therefore, could be distinguished from template-derived proteins that were labeled with ^{14}C. Fractions from the sucrose gradient shown in Fig. 1 were trichloroacetic acid precipitated, and the proteins were identified by electrophoresis on SDS-acrylamide gels. As Fig. 2 demonstrates, both L and NS ^{3}H-proteins were found in fraction 7, which contained the most polymerase activity. NS rebinds much more efficiently than L protein does because L is so easily denatured (Fig. 2B). Fractions 6, 8, and 9 had similar protein composition, as expected (data not shown). The M protein in the complex represents a small percentage of that found in the intact virion and has been considered a contaminant rather than potential polymerase because of previous experiments that demonstrate M protein is not required for transcription. The gradient pellet contained both L and M proteins but no demonstrable NS; unbound L protein aggregates and precipitates out of solution at low ionic strength. Because both L and NS proteins reassociate with the RNP concomitant with reconstitution of the transcriptive complex, either or both could qualify as the polymerase.

Glycerol gradient separation of soluble L

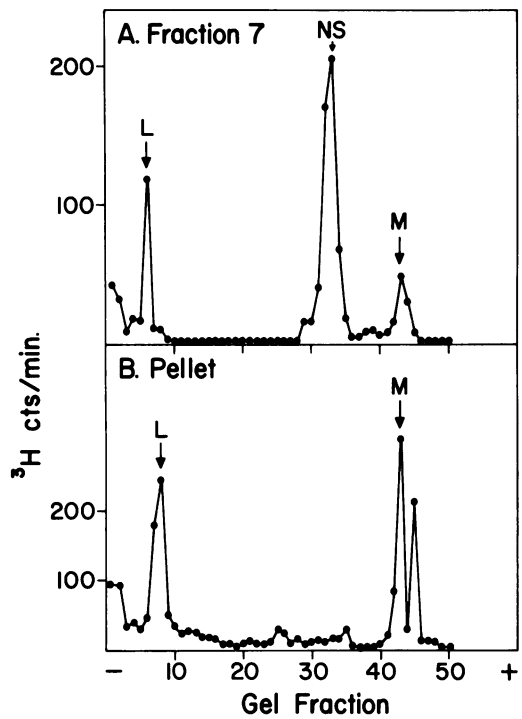

FIG. 2. *SDS-acrylamide gel electrophoresis of proteins from the sucrose gradient shown in Fig. 1. Part of each unincubated fraction that contained significant ^{3}H-protein was precipitated by trichloroacetic acid and examined by electrophoresis on 7.5% acrylamide gels. Panel A shows the ^{3}H-proteins in fraction 7 that contained most of the transcriptive complex. This gel also had a peak of template ^{14}C-labeled N protein at fraction 29. Panel B illustrates the composition of the precipitated, aggregated proteins present in the pellet fraction of the sucrose gradient; the small amount of ^{14}C present in the gradient pellet was not detectable on the gel.*

and NS proteins. The L protein has a molecular weight of ~190,000, whereas that of NS protein is only about 45,000 (20); therefore, an attempt was made to separate the two proteins by velocity sedimentation through a glycerol gradient. ^{3}H-amino acid-labeled virions were treated with Triton-high-salt solubilizer and fractionated by centrifugation, as already described. The supernatant fraction (1.6 ml) was mixed with 0.4 ml of 0.5 M Tris-hydrochloride (pH 7.2) and 50 μg of hemoglobin carrier after which $(NH_4)_2SO_4$ was added to 50% saturation. The mixture was stored at 4 C for 4.5 h and then centrifuged in the SS34 rotor of a Sorvall centrifuge at 15,000 rpm. Under these conditions, the Triton and protein form a pellicle rather than a pellet. The Triton-protein pellicle was dissolved in 0.25 ml of high-salt solubilizer lacking Triton, and 0.1 ml of RSB was added to lower the density. A 0.3-ml portion of this mixture was layered onto a 5.0-ml preformed 10 to 35% glycerol gradient underlaid with a pad of 80% glycerol (Materials and Methods). The gradient contained 0.72 M NaCl to minimize aggregation. Centrifugation was at 220,000 × *g* for 18 h at 4 C in an SW65 rotor. Fractions of 0.5 ml were collected manually into tubes containing 100 μg of hemoglobin carrier and were dialyzed against glycerol column wash containing 0.288 M NaCl to adjust the NaCl concentration. A 0.2-ml portion of each fraction was mixed with 0.05 ml of a standard preparation of ^{14}C-amino acid-labeled template in diluent solution and 0.25 ml of complete polymerase reaction solution containing ^{3}H-UTP. Samples of 0.1 ml each were assayed for polymerase activity, as previously described. The remaining samples were trichloroacetic acid precipitated, mixed with ^{14}C-amino acid-labeled virion marker proteins and the proteins identified by SDS-acrylamide gel electrophoresis.

The glycerol gradient separated the soluble virion proteins into two bands (Fig. 3), one of which contained polymerase, whereas the other was devoid of enzyme activity. Fraction 2, where most of the polymerase activity was located, lacked NS protein but was rich in L protein (Fig. 4). L was also found in fractions 1 and 3, whereas NS protein, as well as G and M proteins, were found scattered in fractions 3 to 8.

Phosphocellulose column separation of L and NS proteins. ^{3}H-amino acid-labeled VS virions were fractionated by Triton-NaCl solubilization and separated by centrifugation as described. The supernatant was dialyzed twice at 4 C against 50 volumes of glycerol column wash containing glycerol, Triton X-100, and

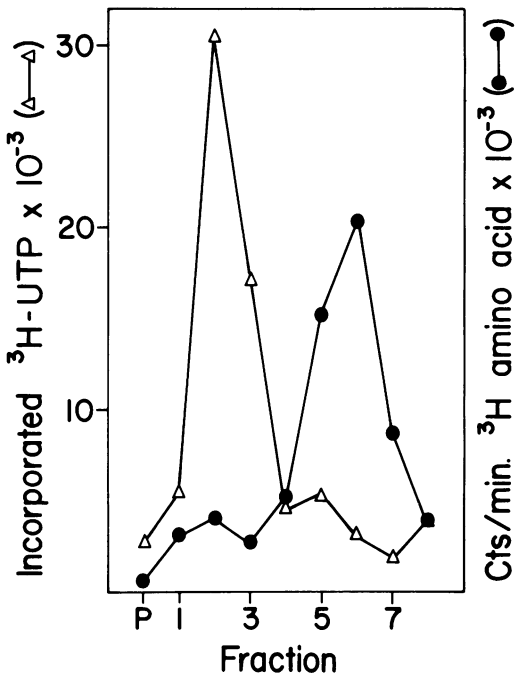

FIG. 3. *Glycerol gradient separation of viral ^{3}H-proteins in the supernatant fraction of VS virions treated with Triton-high-salt solubilizer. Solubilized supernatant proteins were concentrated by $(NH_4)_2SO_4$ precipitation and layered onto a 5-ml, 10 to 35%, glycerol gradient that was centrifuged for 18 h at 225,000 × g. Fractions of 0.5 ml each were collected, and a portion of each was counted to locate the proteins (●). Each fraction was then dialyzed to lower the NaCl concentration to 0.288 M, mixed with polymerase reaction mixture and RNP template, and assayed for polymerase activity by incorporation of ^{3}H-UTP into trichloroacetic acid-insoluble material (Δ). The template, without added supernatant fluid, incorporated 4,513 counts/min in 3 h, and this background value was subtracted from each sample. The top of the gradient is to the right.*

DTT in 0.05 M Tris (pH 7.4). Total dialysis time was 3 h. After addition of 50 μg of hemoglobin carrier, the dialyzed supernatant was loaded onto a 2-ml phosphocellulose column that had been equilibrated with the same glycerol column wash. Fractions of approximately 1 ml were collected manually by placing the end of the effluent tubing directly at the bottom of a test tube containing 100 μg of hemoglobin in 20 μliters of water. Column chromatography was carried out at 4 C and involved step elution of the column, first with 5.0 ml of the glycerol column wash, then with 7.0 ml of column wash containing 0.5 M NaCl, and finally with 7.0 ml of column wash containing 1.0 M NaCl. Individual fractions were then

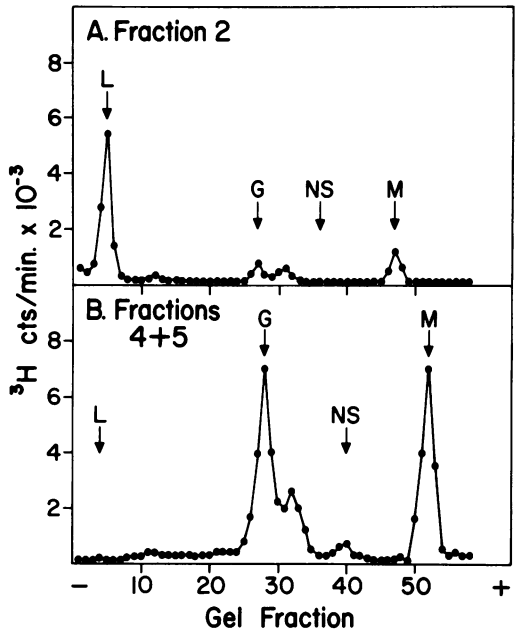

FIG. 4. *SDS-acrylamide gel electrophoresis of ^{3}H-protein fractions taken from the glycerol gradient shown in Fig. 3. Panel A shows the proteins present in fraction 2, representing the material that sedimented most rapidly on the gradient and accumulated on the 80% glycerol pad, whereas Panel B shows proteins present in pooled fractions 4 and 5. The arrows mark the peak fractions of VS virion ^{14}C-proteins co-electrophoresed with the glycerol gradient fractions of the solubilized viral ^{3}H-proteins.*

dialyzed overnight against glycerol column wash made with RSB instead of Tris and containing 0.288 M NaCl. Template was prepared as usual from ^{14}C-amino acid-labeled VS virus and was stored frozen in diluent solution. Phosphocellulose column fractions of 0.3 ml each were mixed with 0.1 ml of template and 0.4 ml of polymerase reaction mix and incubated at 31 C to measure polymerase activity. An unincubated sample served to locate the viral ^{3}H-protein.

Figure 5 shows the elution pattern of labeled viral proteins superimposed upon the polymerase activity profile of the phosphocellulose column. All polymerase activity was recovered in a single peak eluting at the highest salt concentration. The template by itself incorporated 2,234 counts/min during the 3-h incubation period; this background incorporation by the template has not been subtracted and is constant for each fraction assayed, showing that no fraction contains an inhibitor of the enzyme activity. This result is important because fractions 1 to 3 contain higher levels of Triton X-100 than do the remaining fractions.

Unincubated fractions collected from the phosphocellulose column were trichloroacetic acid precipitated and examined by electrophoresis on SDS-acrylamide gels. As Fig. 6 demonstrates, the NS and L proteins were completely separated. NS did not bind to the column but was washed through; fractions containing NS protein were unable to synthesize RNA when added to RNP template. L protein, however, was found in fraction 14 coincident with extensive polymerase activity. Fraction 14 that contained L protein also had a small amount of G and M proteins, but neither of these proteins affects transcription as indicated by the absence of activity in fractions 7 to 9 (Fig. 5). Elution of VS viral solubilized proteins from an identical phosphocellulose column by means of a 0.5 to 1.0 M NaCl gradient was only partially successful in separating the L protein from the small amount of contaminating G and M proteins; once again, however, all polymerase activity co-chromatographed with the L protein. In addition, mixing proteins NS (peak 1) or G and M (peak 2) with L protein (peak 3) produced no detectable increase in the amount of RNA synthesized from template over that produced by L protein and template alone.

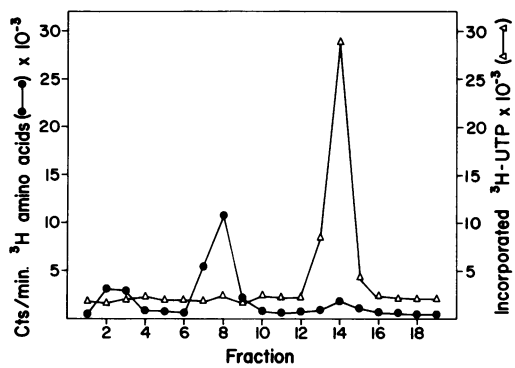

FIG. 5. *Fractionation of the VS virion polymerase on a phosphocellulose column. Viral ^{3}H-proteins solubilized with Triton-high-salt were dialyzed to lower the ionic strength and loaded onto a 2-ml phosphocellulose column. Proteins were eluted by washing the column first with low ionic strength glycerol column wash (fractions 1 to 5), then with the same solution containing 0.5 M NaCl (fractions 6 to 12), and finally with 1.0 M NaCl (fractions 13 to 19). Samples (1.0 ml) were collected, and 50 μliters of each was counted to locate the ^{3}H-protein (●). The fractions were dialyzed and mixed with RNP template and polymerase reaction mixture to assay for polymerase activity (△). The points represent the average ^{3}H-UTP incorporation of duplicate samples incubated for 3 h. Samples incubated for 6 h produced the identical pattern but at higher levels of incorporation.*

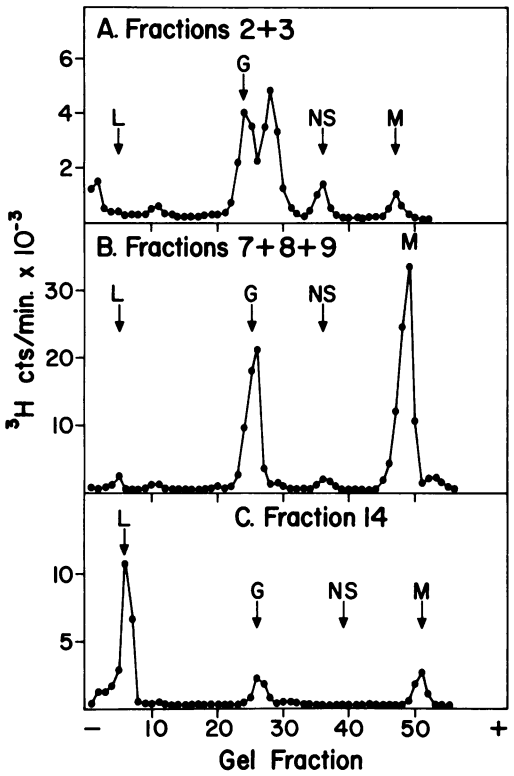

FIG. 6. *SDS-acrylamide gel electrophoresis of proteins fractionated on the phosphocellulose column shown in Fig. 5. Fractions were trichloroacetic acid precipitated, mixed with ^{14}C-labeled viral marker proteins (arrows) and electrophoresed as previously described. Panel A represents the viral protein composition of pooled fractions 2 and 3. Panel B shows the proteins found in pooled fractions 7, 8, and 9, and Panel C show those proteins found in fraction 14, the peak of transcriptase activity.*

The RNA polymers catalyzed by the fraction rich in L protein and deficient in NS protein are complementary to virion RNA. Complete VS virions or reconstituted particles containing both L and NS proteins synthesize only RNA complementary to virion RNA when assayed in vitro (1 to 4). Because both of these systems contain NS as well as L protein, it was conceivable that removal of NS might change the specificity of polymerization so that both complementary and anticomplementary RNA could be made. Therefore, ^{3}H-labeled RNA synthesized in an NS depleted system was analyzed for its capacity to anneal to virion RNA. L protein purified by phosphocellulose chromatography was mixed with a template containing no residual transcriptase activity and was incubated as usual to allow RNA synthesis to proceed. The incubation mixture was centrifuged at 125,000 × *g* to remove the virion RNA that pelleted as part of the RNP, leaving released transcription product in the supernatant. The supernatant ^{3}H-uridine-labeled RNA was mixed with yeast carrier RNA and purified by phenol extraction and ethanol precipitation. Unlabeled virion RNA was prepared by phenol purification and ethanol precipitation of purified virus. The in vitro product was mixed with 2 μg of virion RNA or with 2 μg of yeast RNA and then was annealed at 60 C for 105 min. Annealed product RNA was quantitated by determining the amount of ^{3}H remaining trichloroacetic acid precipitable after digestion with ribonuclease A and T_1.

Only 7% of the product RNA catalyzed by L protein was resistant to ribonuclease digestion before annealing, whereas 100% was resistant after hybridization to virion RNA (Table 1). Therefore, L protein alone can direct the synthesis of RNA that is complementary to virion RNA, indicating that L protein still functions as a transcriptase in the virtual absence of the nucleocapsid NS protein.

Peptide analysis of the L protein. All of the above experiments indicate that L protein is necessary for in vitro RNA synthesis and, therefore, represents a discrete functional unit. Because of the apparent large molecular weight of the L protein, it was originally postulated that L protein could be a precursor of smaller polypeptides or represent aggregated proteins (21). Stampfer and Baltimore (14) have concluded on the basis of tryptic peptide analysis and pactinomycin experiments that L is a unique poly-

TABLE 1. *Hybridization to unlabeled virion RNA of ^{3}H-RNA synthesized in vitro with L protein purified by phosphocellulose chromatography*

Annealing mixture of 25 μliters of ^{3}H-RNA[a] plus 50 μliters of buffer containing	Trichloroacetic acid-insoluble counts/min		RNase resistant (%)
	−RNase[b]	+RNase[b]	
No additions	8,704	600	6.9
Yeast RNA (2 μg)	8,704	593	6.8
Virion negative strand RNA (2 μg)	8,704	8,720	100.0

[a] ^{3}H-RNA synthesized and released from template during a 3-h incubation at 31 C.

[b] Annealing mixture untreated (−RNase) or treated (+RNase) at 22 C for 45 min with 3,674 U of T_1 RNase plus 230 U of RNase A before trichloroacetic precipitation and collection on nitrocellulose filters.

peptide chain, and we have extended their observations by comparison of the CNBr peptide patterns of the purified virion proteins.

Viral proteins were labeled with either ^{14}C- or ^{3}H-methionine, purified by SDS acrylamide gel electrophoresis, eluted from the gel fragments, and digested with CNBr. Because CNBr cleaves peptide bonds adjacent to methionine residues, each peptide, except for the carboxyterminal peptide, should have a single methionine. Therefore, because every peptide should have the same amount of radioactivity regardless of size, peak areas as well as R_f values can be compared. Each purified ^{14}C-labeled polypeptide was mixed with a ^{3}H-labeled purified polypeptide with which it was to be compared; the mixture was digested with CNBr and then analyzed on SDS-acrylamide gels according to the procedure of Swank and Munkres (16). Not enough NS protein was obtained for analysis, but L, G, N, and M were all compared.

Figure 7 shows representative CNBr peptide patterns of VS viral proteins. To simplify the graphs and still present as much data as possible, one peptide profile has been presented in the standard way, and the peptides of the comparison protein, which were co-chromatographed on the same gel, are shown as shaded areas plotted on a different scale. Comparison of the peptide pattern of L with those of G, N, and M proteins demonstrates extensive differences in all four profiles, indicating that these proteins are not derived from cleavage of L, nor is L an aggregate of one or more of these three proteins. The peptide patterns were reproducible, and similar results were obtained if leucine or tyrosine was used as a label instead of methionine.

DISCUSSION

Two criteria have been applied to identify the supernatant viral component that is functionally the transcriptase of VSV: (i) the enzyme, hence the proteins composing it, must rebind to template during reconstitution of the active system and, (ii) the purified protein or proteins constituting the enzyme activity must stimulate RNA synthesis when added to a functional template. The L protein was the only protein that fulfilled both of these requirements. Although both L and NS proteins can reassociate with template during transcriptase reconstitution, L protein was the only purified protein that promoted RNA synthesis when mixed with RNP. Therefore, we conclude that L protein is an essential component of the transcription system and is probably functioning as the transcriptase or as an integral part of it. Addition of NS to either template alone, or to template plus L protein, did not detectably affect the amount of RNA synthesized. However, it must be cautioned that traces of NS

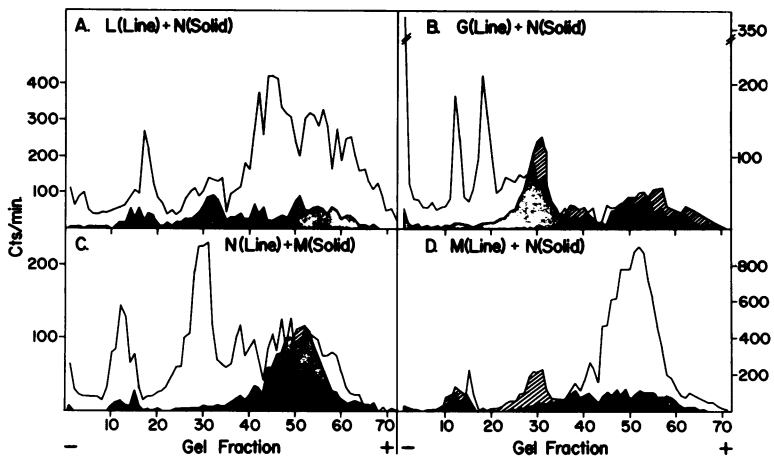

FIG. 7. *Electrophoresis on 12.5% acrylamide gels of CNBr digests of purified VS viral proteins. Electrophoretically purified viral proteins labeled with ^{14}C- or ^{3}H-methionine were cleaved with CNBr producing peptides, each containing one methionine residue. Two proteins, one labeled with ^{14}C and the other with ^{3}H, were mixed, digested with CNBr, and co-electrophoresed on the same gel. However, to simplify the graphs, only one protein of each set has been plotted on the correct scale; the peptide pattern of the co-electrophoresed protein was plotted on a reduced scale and is represented as the shaded area when the peak heights fall below those of the standard protein and as the cross hatched area when the peaks would obscure those of the standard protein. Because all peptides from a single protein should have identical amounts of radioactive label, the area under each peak is as important as the location of the peak. Therefore, a peak containing twice as much label as another peak presumably has twice as many peptides migrating at that position.*

protein (< 10%) often contaminate the template preparations, and it is conceivable that this residual NS protein is sufficient to provide whatever function NS normally performs. The interpretation that both L and NS are absolutely necessary for RNA synthesis seems unwarranted, however, because increasing the concentration of NS by adding purified NS at the same time as L does not further stimulate UTP incorporation. Also, we have shown that addition of NS protein in the absence of L protein has no effect.

Although NS protein may not be required for gross RNA synthesis, such as occurs in vitro, it may be required for the accurate transcription of mRNA that is a prerequisite for viral reproduction in vivo. For instance, NS could function as a regulator to determine the product length or to select which sequences are transcribed. Thus far, we have shown only that RNA synthesis directed by L protein on a template depleted of NS protein is complementary to at least some virion RNA sequences. We have not yet demonstrated that the entire genome is transcribed under these conditions. The observation of Bishop and Roy that only a portion of the genome is transcribed at 37 C, whereas all sequences are copied at 31 C, suggests that some regions of the viral RNA are more readily transcribed than others (2, 4). This observation makes it imperative that the RNA species transcribed from template by isolated L protein in the presence and absence of NS protein be fully characterized before involvement of NS protein in transcription can be discounted. Such analysis of the in vitro RNA products is currently underway in our laboratory.

In addition, we have been able to reconstitute infectivity of VS nucleocapsid template by adding the supernatant fraction containing both L and NS proteins. We are now testing the ability of purified L and NS proteins to restore infectivity to nucleocapsid preparations to determine if both L and NS proteins are required for fidelity and completeness of transcription.

At present we do not know exactly the functional capacity of L protein. Because we estimate about 700 copies of N protein per virion and only around 50 of L protein (unpublished data), we have assumed for stoichiometric reasons that L is the actual polymerase while N has other functions relating to the strict template specificity evident in this system (7). Also, the extremely large size of the L protein is compatible with that of certain *Escherichia coli* proteins that act as RNA polymerases (19). Because of its large size, L protein requires 43% of the genetic information of the virus. The majority of temperature-sensitive mutants isolated for VSV fall into a single complementation group, group I, which appears to have a defective transcriptase at the restrictive temperature (6, 8, 12, 17). This unequal distribution of mutants among different groups can be explained if the majority of mutants have a lesion in the gene coding for the L protein which, because it offers a large target, might be more susceptible to mutations than the genes coding for the smaller proteins. Therefore, identification of the affected protein in group I mutants should provide additional information about the transcriptive process.

Because L protein is the last protein to elute from a phosphocellulose column, one would expect L protein to bind directly to RNA as would be required for a usual polymerase. The ability of L protein to bind to the virion RNA is now being analyzed. NS, on the other hand, does not bind to the phosphocellulose column, suggesting that NS protein interacts directly with N protein. Because NS protein interacts so efficiently with the transcriptive complex both in vivo and in vitro, we have tended to assume that NS protein is in some manner involved with transcription, but there is currently no other evidence to support this hypothesis.

The CNBr peptide data confirms the conclusion of Stampfer and Baltimore (14) that the L protein is indeed a unique gene product. If L were an aggregate, it should contain peptides identical to those in other viral proteins, whereas if it were a precursor, the product proteins should be composed entirely of peptides present in L with the possible exception of the two terminal peptides. The peptide patterns of L, G, M, and N proteins are so different from each other that L cannot be an aggregate of, nor a precursor for, any of these three proteins. Although NS protein has not been similarly mapped, due primarily to insufficient material, the results of the pactinomycin experiments of Stampfer and Baltimore (14) coupled with our demonstration of a specific function for the L protein allow us to assume with some confidence that L is also not a precursor of NS, but is the unique product of a single large gene.

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