JOURNAL OF VIROLOGY, Sept. 1978, p. 560-567
0022-538X/78/0027-0560$02.00/0

Vol. 27, No. 3
Printed in U.S.A.

Rebinding of Transcriptase Components (L and NS Proteins) to the Nucleocapsid Template of Vesicular Stomatitis Virus

MARGARET G. MELLON AND SUZANNE U. EMERSON*
University of Virginia Medical School, Department of Microbiology, Charlottesville, Virginia 22901

Received for publication 7 April 1978

The L and NS proteins of vesicular stomatitis virions (New Jersey serotype) were solubilized with Triton X-100 and high-salt buffer and recombined with purified nucleocapsids under conditions similar to those used to reconstitute transcriptase activity in vitro. The nucleocapsid-bound L and NS proteins were separated from unbound proteins on a glycerol gradient. The rebinding of L and NS proteins mimics the in vivo binding in that at saturation the ratio of L and NS molecules to N molecules is approximately the same as observed in the intact virion. L and NS proteins were separated and added back independently and in combination to the template. The purified NS protein bound to the template in the absence of L protein. However, the L protein binding appeared to depend on the presence of NS protein. The presence of Mg^{2+} and nucleotides, which is required for transcription, was not necessary for the rebinding of L and NS proteins.

Most, if not all, rhabdoviruses carry an RNA-dependent RNA polymerase in the virion (14). In several rhabdoviruses, including the well-characterized vesicular stomatitis (VS) virus, the polymerase activity may be dissociated from the ribonucleocapsid template and the transcription system then reconstituted by recombination of the separated components (7, 5). Under this reconstitution system, three of the five viral proteins have been identified as necessary for transcription (8, 9, 11). These are the major structural protein N and the minor proteins L and NS; the two membrane-associated proteins, G and M, are apparently not required (7, 6). The N protein is probably required as an integral structural component of the template; only the nucleocapsid consisting of N protein tightly complexed to the viral RNA serves as a template for transcription (7, 11). Deproteinized viral RNA is not transcribed in the in vitro system. The L and NS proteins, which have been purified as a complex, active in in vitro transcription (11), almost certainly constitute the transcriptase enzyme. However, mRNA synthesis catalyzed by the transcriptase is a complex process. The mRNA's transcribed in the reconstituted transcription system are capped and methylated at their 5′ ends and polyadenylated at their 3′ ends (1). The capping and polyadenylating activities which modify the mRNA's are so tightly coupled with the transcriptase activity that methods for separating these activities from transcription have so far eluded investigators. Thus, the detailed functions of the L and NS proteins in the overall production of mRNA's remain obscure.

As an approach to understanding the roles of the L and NS proteins, we have sought information on the rebinding of these two proteins to the nucleocapsid of VS virus. In this report we have developed an assay for the rebinding of L and NS proteins of VS virus New Jersey to nucleocapsids and used it to characterize the binding capabilities of the separated L and NS proteins and to determine the stoichiometry of and the requirements for the binding reaction. Since the polymerase rebinds to the template during transcription, our assay conditions are based on those for the in vitro reconstitution of transcriptase activity (7).

MATERIALS AND METHODS

Viruses and cells. The New Jersey serotype of VS virus was originally obtained from the U.S. Department of Agriculture, Beltsville, Md., by R. R. Wagner and was recently classified as the Concan subtype (13). The virus was grown at 31°C in confluent monolayers of BHK-21 cells in 150-mm^2 Corning tissue culture flasks with Dulbecco modified Eagle medium, high glucose, and 10% tryptose broth. Virus was radiolabeled by including 150 μCi of [^{35}S]methionine per ml, 100 μCi of ^{3}H-labeled L-amino acid mixture per ml, or 50 μCi of [^{3}H]leucine per ml in the infecting medium. Cultures were infected at 0.5 to 2 PFU per cell, and virions were harvested at 16 to 18 h postinfection. The overlay medium was clarified by a 10-min centrifugation at 1,000 × *g*, and the virions were pelleted through a layer of 50% glycerol onto a pad of Flouroinert by centrifugation at 80,000 × *g* for 90 min. The virus band was removed from the surface of the Flourinert pad and layered onto a preformed linear 0 to 40% sucrose

gradient containing 1 M NaCl and 1 mM EDTA (7, 10). The band of B virions was collected from the gradient and concentrated by centrifugation at 80,000 × *g* for 90 min. The virus was then centrifuged to equilibrium overnight on a preformed linear 10 to 40% sucrose gradient containing 1 M NaCl and 1 mM EDTA and concentrated again by centrifugation. The final pellet, the harvest of 15 bottles of tissue culture cells (5 to 10 mg), was resuspended in 1 to 2 ml of 0.01 M Tris-hydrochloride (pH 7.4) plus 15% glycerol and stored at −18°C until needed.

Solubilization of viral proteins for rebinding. Viral proteins for rebinding were labeled with [^{35}S]-methionine or a ^{3}H-labeled mixture of amino acids. The virus harvest from approximately five bottles of BHK-21 cells (~3 mg of purified virus in 0.3 ml) was mixed with an equal volume of 2× Triton-high-salt solubilizer (2× HSS = 18.7% glycerol, 1.44 M NaCl, 1.2 × 10^{-3} M dithiothreitol, 3.74% Triton X-100, and 10^{-2} M Tris-hydrochloride, pH 7.4) (7). The solubilized virions were incubated at 31°C for 30 min. The nucleocapsid template was pelleted from the solution by centrifugation at 125,000 × *g* for 2 h, leaving the supernatant fraction containing the solubilized G, M, L, and NS viral proteins. Viral proteins were always solubilized immediately before their use in binding experiments.

Template purification. To completely remove the tightly adhering NS protein, very dilute samples of virus were used to prepare a nucleocapsid template. A 15-bottle harvest of purified virus (5 to 10 mg) labeled with [^{3}H]leucine or [^{35}S]methionine was diluted to 43 ml with 0.01 M Tris-hydrochloride, pH 7.4. The virions were disrupted by mixing with an equal volume of 2× HSS (see above). The nucleocapsids were centrifuged onto a pad of renografin at 90,000 × *g* overnight, collected from the surface of the pad, and then chromatographed on an agarose (Bio-Gel A-5M) column equilibrated with 10% glycerol, 1 M NaCl, 0.1% Triton, 10^{-4} M dithiothreitol, and 0.01 M Tris-hydrochloride, pH 7.4. Approximately 2-ml fractions were collected from the column. The fractions containing the labeled nucleocapsids, which eluted with the void volume, were pooled and centrifuged through a layer of 20% glycerol onto a 100% glycerol pad by centrifugation at 125,000 × *g*. The nucleocapsids were removed along with the 100% glycerol pad and stored at −18°C until used.

Binding assays. Details of each binding assay are given in the figure legends. The standard assay, based on the optimal conditions for transcription, was set up according to the following scheme (7). Since the salt optimum for transcription is ~0.15 M NaCl, the viral proteins, solubilized in 0.72 M NaCl, were diluted 1:4 into the standard assay mixture to give a final NaCl concentration of 0.144 M (80 μl of solubilized proteins in a 0.4-ml final reaction volume). The prereaction mix (1.4 × 10^{-3} M ATP, GTP, and CTP; 8 × 10^{-3} M Mg^{2+}; 1.2 × 10^{-3} M dithiothreitol; and 5 × 10^{-2} M Tris, pH 7.4) was two times the desired concentration of nucleotides and Mg^{2+}, and 0.2 ml of the prereaction mix was included in the 0.4-ml final volume. Various volumes of purified template (in 100% glycerol) and 0.01 M Tris-hydrochloride, pH 7.4, made up the remaining 0.12 ml of the final reaction volume.

Glycerol gradients. Linear 15 to 35% glycerol gradients (5 ml) were formed by using a custom gradient maker. The 15 and 35% glycerol solutions were made by diluting either 3 or 7 ml of glycerol to 20 ml with glycerol gradient mix (7 × 10^{-4} M ATP, CTP, and GTP, 4 × 10^{-3} M magnesium acetate, 0.144 M NaCl, 2% Triton, and 6 × 10^{-4} M DTT in 0.01 M Tris, pH 7.4). ATP, CTP, and GTP were included in gradients only in early experiments. Once it was determined that nucleotides were not required for binding, they were omitted from the gradients.

SDS-polyacrylamide gel electrophoresis. Protein samples were mixed with 100 μg of hemoglobin carrier and 1 volume of 40% trichloroacetic acid and centrifuged at 2,000 × *g* to pellet the precipitated protein. The pellet was washed one or two times with acetone and dissolved in a solution of 0.01 M Tris-hydrochloride with 8 M urea, 1% sodium dodecyl sulfate (SDS), and 1% mercaptoethanol. After boiling for 2 min, the samples were subjected to electrophoresis on 7.5% SDS-phosphate acrylamide gels. Gel slices were incubated with 0.5 ml of Nuclear-Chicago solubilizer (NCS) for 2 h at 50°C and counted in LSC Complete toluene-based scintillation fluid.

Preparation and recombination of the L and NS protein fractions. The preparation of L and NS proteins from VS New Jersey serotype virus is a modification of the published procedure using VS Indiana serotype virus (8, 9). Approximately 1 ml (~10 mg) of VS New Jersey virus labeled with [^{35}S]methionine was diluted to 10 ml and mixed with an equal volume of 2× HSS (see above). The nucleocapsids were removed by centrifugation for 2 h at 125,000 × *g*. The supernatant was mixed with 5 to 10 ml of phosphocellulose previously equilibrated with Tris, pH 7.2, and then dialyzed overnight against column buffer (75 ml of glycerol, 3 ml of 20% Triton, 9 mg of DTT, and 222 ml of 5 × 10^{-2} M Tris-hydrochloride, pH 7.2) containing 0.1 M NaCl. The phosphocellulose and ^{35}S-labeled supernatant protein mixture was then transferred to a 12-ml column, drained, and washed with more 0.1 M NaCl column buffer; the flow-through was saved and used later. The phosphocellulose column was eluted with 0.5 M NaCl column buffer, and the peak fractions containing the ^{35}S-labeled proteins were called the L-protein fraction. Because of the instability of the L protein, the L-protein fraction was always used immediately.

The reserved flow-through from the phosphocellulose column, containing primarily NS and G proteins, was applied to a 4- to 6-ml DEAE-cellulose column, and the column was washed with 0.1 M NaCl column buffer. The NS protein was then eluted with 0.4 M NaCl in column buffer. To completely remove L protein, it was necessary to dialyze the NS-protein fraction overnight against 0.1 M NaCl column buffer and to process the NS protein through another cycle of phosphocellulose and DEAE-cellulose chromatography. Purified NS protein was stored at −18°C.

When used in binding assays, the ^{35}S-labeled L- and NS-protein fractions were recombined with ^{3}H-labeled purified template. The binding assay mixture with both L and NS proteins contained 0.06 ml of L-protein fraction, 0.08 ml of NS-protein fraction, 0.025 ml of template, 0.035 ml of 0.01 M Tris, pH 7.4, and 0.2 ml

of prereaction mix (see above), pH 7.4. When L- or NS-protein fraction was added separately, either 0.08 ml of 0.4 M NaCl column wash or 0.06 ml of 0.5 M NaCl column wash, respectively, was added to maintain the proper salt concentration. The binding reactions were incubated at 31°C for 30 min. The template-bound proteins in each preparation were isolated on glycerol gradients and analyzed on SDS-polyacrylamide gels.

Stoichiometry of L and NS proteins bound to template. The relative distribution of label in L, NS, and N proteins was obtained from SDS-polyacrylamide gels. Small samples of intact virus were dissolved in gel sample buffer and applied directly to the gel. Template and rebound proteins were isolated on glycerol gradients, trichloroacetic acid precipitated, and then analyzed on gels. In the case where intact virions were compared to the dissociated and immediately recombined supernatant and pellet fraction, the virus was labeled with a ^{3}H-labeled mixture of amino acids to uniformly label all viral proteins. The relative amount of radioactivity in L, NS, and N proteins was determined and divided by the molecular weights for L, NS, and N proteins (190,000, 40,000, and 50,000, respectively) to give molecular ratios. The molecular ratio for N was divided into 2,000, and the molecular ratios for L and NS proteins were multiplied by this conversion factor. The final values represent the number of L and NS molecules per 2,000 N molecules on the gel.

When purified template and solubilized viral proteins were recombined, the template was labeled with [^{35}S]methionine, and the solubilized viral proteins were labeled with a ^{3}H-labeled mixture of amino acids. In this case the specific activities of the ^{3}H-solubilized proteins and the ^{35}S-labeled N protein were calculated based on a Lowry protein assay, and the specific activities of the three proteins were then used to determine the stoichiometry of L and NS proteins per 2,000 N molecules.

Chemicals. [^{3}H]leucine and [^{35}S]methionine were obtained from Amersham/Searle, Arlington Heights, Ill., and the ^{3}H-labeled L-amino acid mixture was from New England Nuclear Corp., Boston, Mass. Whatman P11-cellulose and microgranular DEAE-cellulose DE52 were purchased from Whatman Biochemicals (Maidstone, England). Renografin was obtained from E. R. Squibb & Sons, Princeton, N.J., and Flouroinert was from Instrumentation Specialties Co., Lincoln, Neb. LSC Complete scintillation fluid was from Yorktown Research, Hackensack, N.J. Agarose (Bio-Gel A-5 M), acrylamide, and *N,N*-methylenebisacrylamide were obtained from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Solubilized viral proteins rebound to the template. The L and NS proteins were solubilized along with the M and G proteins by treating whole ^{35}S-labeled virus with Triton X-100 and high salt (0.72 M NaCl) and pelleting out the nucleocapsids. This supernatant fraction was used immediately in binding assays. Purified template, consisting of only the N protein and viral RNA, was prepared from another batch of ^{3}H-labeled virus by standard methods (see Materials and Methods).

The purified ^{3}H-labeled template was recombined with the ^{35}S-labeled supernatant proteins under transcription assay conditions. Final concentrations of Mg^{2+}, NaCl, ATP, GTP, and CTP were optimal for transcription, but UTP was omitted to prevent transcription from occurring (8). The reaction was incubated at 31°C for 30 min, and then the entire 0.4-ml reaction mixture was layered on a 15 to 35% glycerol gradient made up in a solution similar to the reaction mixture. Figure 1 shows the distribution of ^{35}S and ^{3}H label in the glycerol gradient. The template along with bound ^{35}S-labeled proteins sedimented to the middle of the gradient. The unbound proteins remained at the top. Aggregated template (30 to 50% of total) and proteins (~10% of total) sedimented through the gradient and pelleted. When supernatant proteins were applied to the gradient in the absence of template, no proteins were found in the middle of the

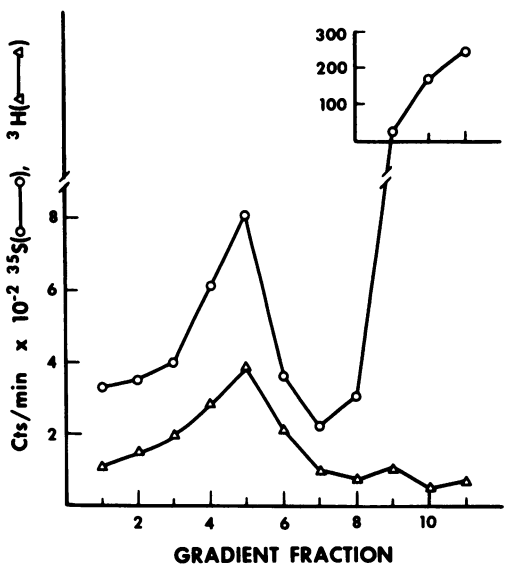

FIG. 1. *Glycerol gradient of the binding reaction mixture. High-salt-solubilized VS New Jersey viral proteins labeled with [^{35}S]methionine and template labeled with [^{3}H]leucine were prepared as described in the text. The binding reaction mixture contained 80 μl of the solubilized ^{35}S-labeled viral proteins, 70 μl of template, 50 μl of 0.1 M Tris, pH 7.4, and 0.2 ml of prereaction mixture (1.4 × 10^{-3} M ATP, CTP, and GTP, 1.2 × 10^{-3} M dithiothreitol, and 5 × 10^{-2} M Tris-hydrochloride, pH 7.4). After a 30-min incubation at 31°C, the entire 0.4-ml reaction mixture was layered on a 15 to 35% glycerol gradient (see text) and centrifuged at 125,000 × g for 1 h at 4°C. Portions (50 μl) of 0.5-ml gradient fractions were counted and plotted. The direction of sedimentation is from right to left.*

gradient (data not shown).

To determine which of the four solubilized proteins had bound to the template, the gradient fractions containing the cosedimenting ^{35}S- and ^{3}H-labeled proteins were precipitated with trichloroacetic acid and analyzed on an SDS-polyacrylamide gel. Despite the fact that M and G proteins were present in much greater abundance than the L and NS proteins in the solubilized supernatant fraction, only traces of M and G proteins were found cosedimenting with template (Fig. 2). Essentially only the L and NS proteins rebound to template.

In vitro rebinding of L and NS proteins mimicked the stoichiometry of intact virions. In view of the marked tendency of the L protein to aggregate in vitro, it is important to show that the observed cosedimentation of L and NS proteins with template represents a rebinding of L and NS proteins to specific sites on the template. If the L and NS proteins are rebinding to specific sites on the template, it should be possible to saturate the available sites on the template to approximately the same extent as in undisrupted virions.

A series of binding reactions was set up with constant amounts of ^{35}S-labeled supernatant proteins and decreasing amounts of ^{3}H-labeled template, in effect increasing the concentration of L and NS proteins relative to the template. Each of the reaction mixtures was applied to a glycerol gradient, and the template-bound proteins were analyzed on SDS-polyacrylamide gels. The amounts of L and NS proteins bound were normalized to the N protein on the gel. The amounts of bound L and NS proteins increased linearly with increasing relative concentrations of L and NS proteins and then reached a plateau, showing that sites on the template can be saturated with L and NS proteins (Fig. 3).

Further evidence that the binding is significant was obtained by comparing the number of L and NS proteins rebound to the template and

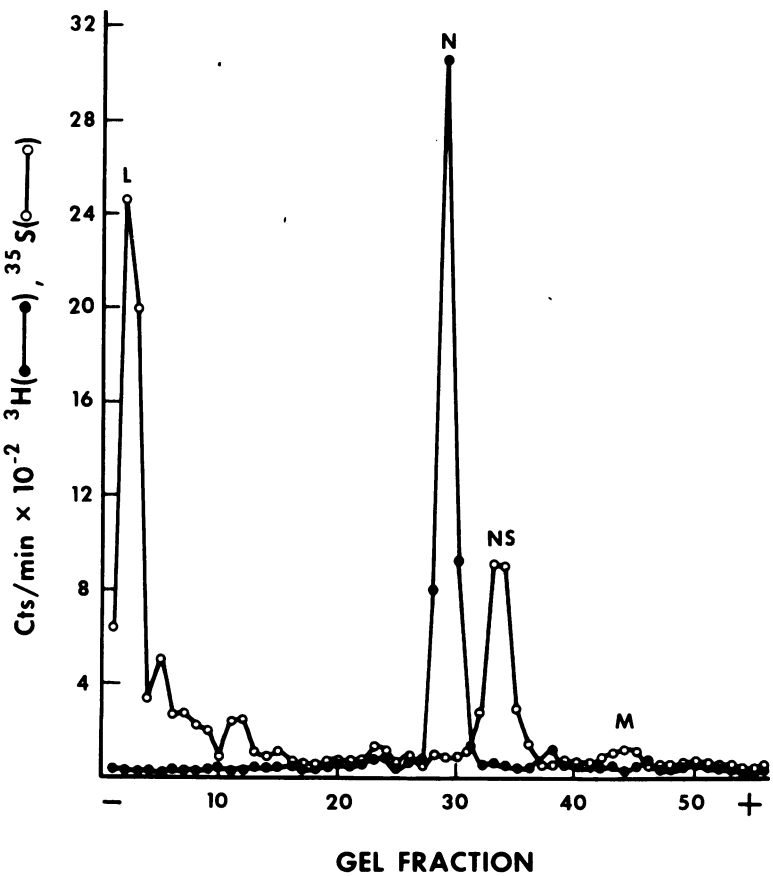

FIG. 2. *SDS-polyacrylamide gel electrophoresis of template-bound proteins. Fractions 4, 5, and 6 from the gradient represented in Fig. 1 were pooled, and the proteins were analyzed on SDS-phosphate polyacrylamide gels. The template was labeled with ^{3}H, and the solubilized supernatant proteins were labeled with ^{35}S.*

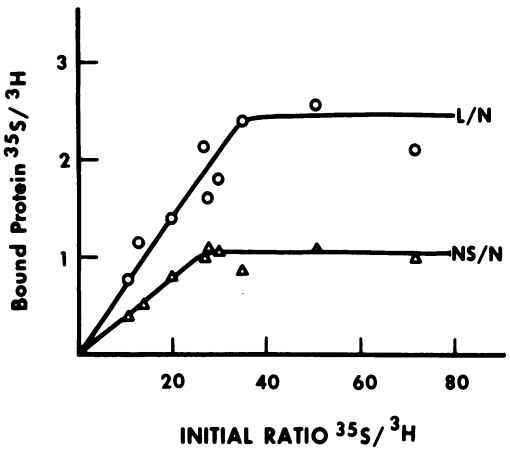

FIG. 3. *Binding of L and NS proteins to template. A series of binding assays was set up and processed as described in Fig. 1 and 2. Each tube contained 0.2 ml of the prereaction mixture, 80 μl of the ^{35}S-solubilized viral proteins, and decreasing volumes (80 to 10 μl) of ^{3}H-labeled template. Sufficient 0.01 M Tris-hydrochloride, pH 7.4, was added to each tube to bring the total volume to 0.4 ml. After separation of bound from unbound ^{35}S-labeled proteins on glycerol gradients, the pooled gradient fractions containing the rebound L and NS proteins were analyzed on SDS-polyacrylamide gels. The counts of ^{35}S in the L or NS proteins were normalized to the counts of ^{3}H-labeled N protein on each gel. These values were plotted against the initial ratio of ^{35}S-labeled solubilized supernatant proteins/^{3}H-labeled template. This ratio was determined by counting small samples of each reaction mixture before layering on the glycerol gradients.*

the number of L and NS proteins contained in the intact virion. If the number of L and NS proteins rebound to template in vitro greatly exceeded the number of L and NS proteins normally carried by the virion, a nonspecific aggregation might be suspected. On the other hand, if under saturating conditions the numbers of L and NS proteins rebound to the template were approximately equivalent to the virion values, specific rebinding would be supported.

For these experiments a ^{3}H-labeled amino acid mixture was used to uniformly label all the viral proteins. A sample of virus was dissociated with the 2× HSS and separated by centrifugation into a supernatant and pellet fraction. The solubilized supernatant fraction and either 0.1 or 0.05 ml of the pellet fraction which contained the template were immediately recombined under standard binding assay conditions and applied to a glycerol gradient. The template-bound proteins were then analyzed on SDS-polyacrylamide gels. Undissociated virus from the same preparation was also analyzed on an SDS-polyacrylamide gel. The numbers of L and NS molecules, arbitrarily expressed per 2,000 N molecules, were determined from each gel as detailed in Materials and Methods. Where the template was not saturated, the number of L and NS molecules rebound per 2,000 N molecules was less than the number of L and NS proteins found in the intact virion (Table 1).

However, in the case where the template was saturated, there was excellent agreement between the numbers of L and NS molecules bound in vitro and in vivo (Table 1). In this experiment L and NS proteins were rebound to purified template and the concentration of L and NS proteins was shown to be saturating. The amount of L and NS proteins rebound per 2,000 N molecules in this experiment approached closely the number determined for the intact virion. These results suggest that the rebinding is specific in that at saturation the rebinding of L and NS proteins mimicked the binding observed in the intact virion.

NS bound to template in the absence of L protein. It was of interest to examine the separate binding capabilities of the L and NS proteins. The NS protein can be purified from the mixture of solubilized viral proteins by several cycles of column chromatography (see Materials and Methods). A series of binding assays was set up with increasing concentrations of NS protein and constant amounts of template. An SDS-polyacrylamide gel of the purified NS fraction showed 95% of the fraction to consist of NS protein. Purified NS protein bound to the template in the absence of L protein (Fig. 4). Figure 5 shows that the binding reaction was linear with increased protein concentration and that NS protein saturated the template.

TABLE 1. *Numbers of L and NS molecules bound per 2,000 N molecules*[a]

Proteins	Mol wt	Intact virions[b]	Rebound L and NS proteins		
			Subsaturation[c]		Saturation[d]
			0.1 Template	0.05 Template	
L	190,000	131	47	76	142
NS	40,000	267	160	224	230

[a] See the text for calculations.

[b] Intact virions were solubilized and analyzed directly on polyacrylamide gels.

[c] Same preparations as intact virion. The virus was solubilized, and supernatant and pellet fractions were obtained by centrifugation. Portions (80 μl) of the supernatant protein and 0.1 or 0.05 ml of the pellet fraction were immediately recombined in the standard binding assay.

[d] Purified template and solubilized proteins prepared from other virus samples were recombined under saturating conditions in a standard binding assay.

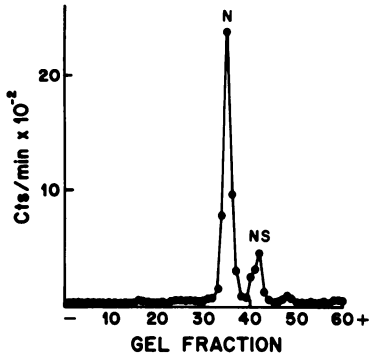

FIG. 4. *SDS-polyacrylamide gel electrophoresis of template-bound NS protein from a binding assay containing saturating amounts of NS protein. Binding assay conditions are described in the legend to Fig. 5.*

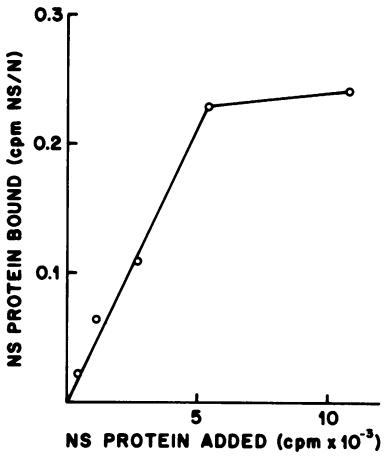

FIG. 5. *Binding of NS protein to the nucleocapsid template. NS protein labeled with ^{35}S was purified by chromatography as described in the text and dialyzed against glycerol column wash with 0.1 M NaCl. Template labeled with ^{35}S was prepared as described in the text. Serial dilutions of the NS protein were made in 0.1 M NaCl column wash. A 0.1-ml amount of NS protein from each of the dilutions was added to 20 μl of template, 60 μl of 1× HSS, 20 μl of Tris, and 0.2 ml of prereaction mix (see text). The binding reactions were incubated at 31°C for 30 min. The template and bound proteins were isolated on glycerol gradients and analyzed on SDS-polyacrylamide gels. The counts in the NS protein bound were normalized to the counts in the N protein on the gels and plotted against the ^{35}S counts per minute in 20 μl of each of the dilutions of purified NS added to the assay.*

L protein binding depended on the presence of NS protein. In contrast to the binding capability of NS protein, the L protein did not appear to bind to template in the absence of NS protein. For this experiment L- and NS-protein fractions were prepared by column chromatography as described in Materials and Methods. The L-protein fraction was free of NS protein but contained substantial amounts of M protein. However, since the M protein did not rebind to the template under these conditions, further manipulations to purify the extremely unstable L protein were not attempted. The NS fraction used was at least 95% pure.

The L- and NS-protein fractions were enzymatically active when combined and tested in transcription assays. However, when tested alone each fraction was inactive, showing that the fractions were free of cross-contamination (data not shown).

The L and NS fractions were added either alone or in combination to purified template. Table 2 shows that when the L-protein fraction was added alone, no binding to the template was seen. However, when L protein was added in the presence of NS protein, L protein did bind to the template. Thus, the binding of L protein seemed to be dependent on the presence of NS protein. Similar amounts of NS protein bound to template in the presence or absence of L protein.

Requirements for binding. Although the binding assay was originally developed by using conditions optimal for transcription, further experiments were done to see to what extent these conditions were actually required. Table 3 compares binding of L and NS proteins under saturating conditions in the presence and absence of Mg^{2+} and nucleotides. The omission of Mg^{2+} and nucleotides did not significantly affect the amount of binding of the L and NS proteins to the template. Thus, Mg^{2+} and nucleotides were not required for binding L and NS proteins.

Another experiment was done to determine whether the binding reaction would occur at low temperature. After mixing the components under saturating conditions, the binding reaction was left in the ice bucket instead of being incubated at 31°C. Similar amounts of L and NS proteins bound at 4 and 31°C (Table 3). Thus,

TABLE 2. *Binding of L and NS proteins separately and in combination to purified template*

Fractions added[a]	Proteins bound ($^{35}S/^{3}H$)[b]	
	L/N	NS/N
L	<0.005[c]	
NS		0.232
L + NS	0.238	0.249

[a] ^{35}S-labeled L and NS fractions were prepared and recombined with ^{3}H-labeled purified template as described in the text.

[b] Template-bound proteins isolated on glycerol gradients were analyzed on SDS-polyacrylamide gels.

[c] No L protein was detected on the gel.

TABLE 3. *Requirements for the binding of L and NS proteins*

Reaction mixture	Incubation temp[a] (°C)	Proteins bound ($^{35}S/^{3}H$)[b]	
		L/N	NS/N
Complete[c]	31	2.41	0.80
$-Mg^{2+}$, ATP, GTP, CTP	31	2.22	0.78
Complete	31	2.59	1.08
Complete	4	2.54	0.86

[a] After incubation at the indicated temperature, all gradients were loaded in a cold room and centrifuged at 4°C.

[b] Template-bound proteins isolated on glycerol gradients were analyzed on SDS-polyacrylamide gels.

[c] ^{35}S-labeled solubilized viral proteins were prepared as described in the text and recombined under standard assay conditions. All glycerol gradients correspond to prereaction mixtures with respect to nucleotides and Mg^{2+}.

unlike transcription, the binding reaction occurred at 4°C.

DISCUSSION

The reconstitution of transcriptase activity by recombination of template and soluble L and NS proteins implies that these proteins rebind to template in a functionally significant manner. Therefore, the assay to detect this binding is based directly on the conditions for reconstitution of transcriptase activity in vitro (7). The greatest difficulty in developing an assay for rebinding is the denaturation and aggregation of the reaction components, especially the L protein. The assay reported here circumvents this problem by isolating the nucleocapsid with bound proteins on glycerol gradients through which aggregated materials pellet. The amount of bound L and NS proteins detected by the assay saturates with approximately the same number of L and NS molecules per 2,000 N molecules as are present in the intact virion, indicating that the binding detected is a specific rebinding, similar to that which occurs in vivo. In addition, these results suggest that the sites on the template available for binding L and NS proteins are probably fully occupied in the packaged virion.

In determining the numbers of L and NS molecules bound in the intact virion, it was always noted that the numbers of NS molecules were significantly greater than the numbers of L molecules. Of course, such calculations depend on the still unsatisfactory molecular weight assignments for L and NS proteins. The values listed in Table 1 (131 molecules of L and 267 molecules of NS per 2,000 N molecules) were calculated by using 190,000 and 40,000 as the molecular weights for L and NS proteins and result in an NS:L stoichiometric ratio of 2:1. This ratio can be compared to the NS:L ratio of 1:1 reported for intact virions (and the in vitro purified polymerase) by Naito and Ishihama (11). These authors also used VS New Jersey virus and molecular weights of 40,000 to 45,000 for NS protein and 190,000 for L protein. Although reliable stoichiometric ratios await better molecular-weight assignments, especially for NS protein, our data suggest some caution is necessary in accepting the 1:1 stoichiometric ratio for NS to L protein in the intact virion.

When NS and L proteins are solubilized from the virus, it is impossible to determine to what extent they exist in solution as either individual proteins or as L:NS complexes. It is clear that when using the solubilized viral proteins as a source of L and NS proteins, both L and NS are bound to template, but analyzing the binding from such a complex, and as yet uncharacterized, mixture is difficult. Our experiments adding NS and L proteins separately to the template are a start in sorting out the binding capabilities of L and NS proteins. The results with the purified NS protein show that NS protein binds to the template directly in the absence of L protein, i.e., the template must have an accessible binding site for NS protein. However, the binding of L protein appears to be dependent on the presence of NS protein. Since the inherent instability of L protein is accentuated by the manipulations necessary to separate L protein from NS protein, this observation may simply reflect the stabilization of L protein by NS protein. However, since L and NS proteins can bind to each other (11), it is possible that L protein may bind indirectly to template via NS protein. Alternatively, the NS protein may modify the template in some way (e.g., exposing sites on the RNA) which allows L protein to bind directly to template.

Our data on the numbers of bound L and NS molecules have been arbitrarily calculated per 2,000 N molecules, not per virion. However, since reported values for the numbers of N protein per virion range from 1,000 to 2,000 (6, 12), we would roughly estimate 65 or 130 L molecules and 134 or 267 NS molecules per virion. These numbers may be compared to the probable number of promoter sites per genome. A single promoter site per genome is consistent with the sequential transcription of VS virus genes deduced from UV-inactivation studies (4, 2, 3), but, at most, the VS virus genome could reasonably be expected to have five or six promoter sites, one for each gene. Since we detect many more L and NS molecules packaged per virion than could be bound to the one to six promoter sites,

it is likely that the nucleocapsid has another class of binding sites for the polymerase. These may be "reserve" sites, which allow the virion to package an extra supply of polymerase molecules. Experiments in which the transcription of solubilized whole virus can be stimulated at least threefold by the addition of extra purified template (unpublished data, S.U.E.) confirm the suggestion that VS virus packages more polymerase than can be used.

The two classes of binding sites may be quite different. The promoter binding sites are probably restricted to specific sequences of RNA at one or a few sites on the genome. The reserve binding sites may be determined by more general interactions with RNA and/or N protein and may be distributed all along the nucleocapsid. Since our reactions were done under transcriptase conditions, rebinding to both sites probably occurs, but the characteristics of the reaction are dominated by binding to the more numerous reserve sites. Further experiments, including the binding of polymerase to naked genomic RNA, are needed to fully characterize the two types of binding.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants AI-11722 (S.U.E.) and AI-05292 (M.G.M.) from the National Institute of Allergy and Infectious Diseases. 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. The nature of the RNA products synthesized *in vitro* by subviral components of vesicular stomatitis virus. Virology **71**:230–241.
2. **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.
3. **Ball, L. A.** 1977. Transcriptional mapping of vesicular stomatitis virus in vivo. J. Virol. **21**:411–414.
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. **Bishop, D. H. L., S. U. Emerson, and A. Flamand.** 1974. Reconstitution of infectivity and transcriptase activity of homologous and heterologous viruses: vesicular stomatitis (Indiana serotype), Chandipura, vesicular stomatitis (New Jersey serotype), and Cocal Viruses. J. Virol. **14**:139–144.
6. **Bishop, D. H. L., and P. Roy.** 1972. Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. J. Virol. **10**:234–243.
7. **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.
8. **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.
9. **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.
10. **Hunt, D. M., S. U. Emerson, and R. R. Wagner.** 1976. RNA^- temperature-sensitive mutants of vesicular stomatitis virus: L-protein thermosensitivity accounts for transcriptase restriction of group I mutants. J. Virol. **18**:596–603.
11. **Naito, S., and A. Ishihama.** 1976. Function and structure of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. **251**:4307–4314.
12. **Nakai, T., and A. F. Howatson.** 1968. The fine structure of vesicular stomatitis virus. Virology **35**:268–281.
13. **Reichmann, M. E., W. M. Schnitzlein, D. H. L. Bishop, R. A. Lazzarini, S. T. Beatrice, and R. R. Wagner.** 1978. Classification of the New Jersey serotype of vesicular stomatitis virus into two subtypes. J. Virol. **25**:446–449.
14. **Wagner, R. R.** 1975. Reproduction of rhabdoviruses, p. 27–30. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 4. Plenum Publishing Corp., New York.