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Dissociation and Reconstitution of the Transcriptase and Template Activities of Vesicular Stomatitis B and T Virions

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Transcriptase activity was dissociated from vesicular stomatitis virions by high-ionic-strength buffer containing Triton X-100. Considerable enzyme activity could be restored by recombining inactive sedimentable and nonsedimentable virion fractions. Reconstituted transcriptase activity was dependent on the presence of all four nucleoside triphosphates and the concentration of heat-labile molecules in both supernatant and pellet fractions. Lower NaCl concentrations removed ~46% of virion protein, but did not release transcriptase activity from the pellet fraction, nor could incorporation of ^{3}H-uridine-5'-triphosphate by complete virions be increased by adding soluble transcriptase. Evidence that the virion nucleocapsid is the transcription template was provided by finding that the pellet contained predominantly virion core nucleoprotein, ribonucleic acid, and homogeneous nucleocapsid coils when viewed by electron microscopy. Removal of envelope G and M proteins by Triton and low-salt buffer without decreasing nucleocapsid polymerase activity indicates that neither G nor M protein is necessary for transcription. Additional data are required to determine whether the minor nucleocapsid proteins L or NSl, or both, which are at least partially solubilized in high-salt buffer, are the transcriptase. Preliminary data suggest that the major N nucleoprotein, which was not solubilized by high-salt buffer, is also required for transcription. Defective T virions contained at least as much transcriptase per weight as did B virions, as determined by restoration with T supernatant fluids of transcription function to B nucleocapsid template. However, the T nucleocapsid would not serve as template for B or T transcriptase, a finding which is interpreted as evidence of T template defectiveness. The presence of defective T nucleocapsids did not interfere with B or T transcriptase function reconstituted with B template.

Infectious virions of vesicular stomatitis (VS) virus are bullet-shaped (B) particles consisting of an enveloped nucleocapsid core, ~3.5 μm in length, which contains a single ribonucleic acid (RNA) strand of ~3.6 $\times$ 10^6 daltons (9, 14, 15). The VS-B virions contain an RNA-dependent RNA polymerase which can be activated by nonionic detergents, such as Triton N-101 (2). This polymerase functions only with VS nucleocapsid as template and catalyzes only the synthesis of messenger RNA strands complementary to VS virion RNA (2-5). Therefore, this polymerase can be properly designated a transcriptase. Virtually all populations of VS virions released from infected cells contain variable amounts of defective, truncated (T) particles, which are approximately one-third the length of the infectious B particles, and contain roughly one-third the amount of RNA (7, 19). Bishop and Roy (5) have found that defective T particles are devoid of transcriptase activity. However, B and T virions contain the same five proteins in approximately similar proportions (11, 17). Two of these proteins, spike glycoprotein G and matrix protein M (formerly designated S), comprise the envelope; the three proteins identified in the nucleocapsid cores are the major nucleoprotein N and the minor proteins L and NS1 (6, 11, 14, 18, 19).

The present studies describe a method for gently removing transcriptase activity from virion cores and for reconstituting enzyme function by recombining soluble transcriptase with nucleocapsid cores. The ultimate objectives of these continuing studies are to identify and characterize the transcriptase protein or proteins, as well as to delineate its products and mechanism of action.

MATERIALS AND METHODS

Chemicals and radiochemicals. 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. Triton X-100 and pancreatic ribonuclease A were obtained from Sigma Chemical Co., St. Louis, Mo., and T1 ribonuclease was from Worthington Biochemical Corp., Freehold, N.J. ^{3}H-leucine (50 Ci/mmole), ^{3}H-tyrosine (50 Ci/mmole), ^{3}H-uridine-5'-triphosphate (^{3}H-UTP; 15 to 17.4 Ci/mmole), and ^{3}H-5-uridine (28 Ci/mmole) were from Schwarz/Mann, Orangeburg, N.Y. ^{14}C-protein hydrolysate (57 mCi/matom of carbon) was from Amersham/Searle, Arlington Heights, Ill.

Viruses and cells. Cultivation of the Indiana strain of VS virus has been described previously (17, 19). Unless otherwise stated, VS virions were grown on L cells, in Eagle basal medium (BME) diluted 1:50 with Earle's balanced salt solution (BSS) and containing 0.25 μCi of ^{14}C-amino acid hydrolysate per ml, to label viral proteins. The ^{14}C label served as an internal marker during subsequent fractionation of the virion protein and provided a simple means of quantitating any losses and of standardizing samples so that polymerase activities in different fractions could be normalized and compared directly. After 8 hr of infection, in the presence of 4.5 ml of labeling media, 1 ml of minimal essential medium containing 3% fetal calf serum was added, and incubation was continued for 10 more hr. Virus was harvested from the media, which were clarified by centrifugation at 1,000 $\times$ *g* to remove cellular debris. The media were then centrifuged at 60,000 to 80,000 $\times$ *g* in an SW25.1 or SW27 rotor to pellet the virions through a 50% glycerol pad made by diluting one volume of glycerol with one volume of BSS. The pellets were resuspended in BSS, sonicated to disperse any aggregates, and layered on top of a preformed, linear 0 to 40% sucrose gradient. This gradient removes cellular membrane fragments from the virions and allows separation of B and T particles (7). After centrifugation in an SW25.1 rotor for 90 min at 35,000 $\times$ *g*, the visible B or T band was removed by side puncture of the tube, diluted with BSS, and the virions were repelleted in an SW50L rotor at 80,000 $\times$ *g* for 60 min. The purified virions were resuspended in reticulocyte standard buffer (RSB), containing 0.01 M KCl, 0.0015 M $MgSO_4$, and 0.01 M tris(hydroxymethyl)aminomethane (Tris; *p*H 7.4), with DTT and stored at 4 C until assayed for polymerase activity. This purification procedure yielded virions with optimal polymerase activity.

Infectivity of B virions was determined by assay of plaque-forming units (PFU) on monolayers of L cells (7, 17).

Solubilization of virion proteins. Virus particles in RSB were disrupted by addition of an equal volume of 2X-concentrated, Triton-high-salt solubilizer composed of 3.74% Triton X-100 to solubilize the membrane, 1.2 $\times$ 10^{-3} M DTT and 18.7% glycerol to stabilize the polymerase, and 1.44 M NaCl to solubilize certain of the viral proteins, including the transcriptase. Because of the twofold dilution with the RSB virus sample, the final concentration of NaCl during the solubilization process was 0.72 M. A Triton-low-salt solubilizer with 60% the amount of NaCl, but otherwise identical to the high-salt solubilizer, was also used. Samples in solubilizer were incubated at 30 C for 45 to 60 min. Fractionation of virion components into soluble and particulate fractions was achieved by centrifugation of the samples in an SW50L rotor at 125,000 $\times$ *g* for 90 min. The Triton-high-salt supernatant fluid (0.72 M NaCl) was removed and diluted with one and one-half volumes of RSB to give a NaCl concentration of 0.288 M; the corresponding pellet was resuspended in a diluent solution made by mixing one volume of Triton-high-salt solubilizer (0.72 M NaCl) with one and one-half volumes of RSB so that the composition of the pellet and supernatant solvents were identical. The Triton-low-salt pellet (0.43 M) was also resuspended in diluent; however, the 0.43 M supernatant fluid was diluted with RSB containing 0.2 M NaCl so that the final NaCl concentration was also 0.288 M. Virons must be treated with nonionic detergents before the transcriptase can be assayed (2). Therefore, prior to assay, unfractionated virions were suspended in diluent solution with Triton to activate the particles; the assay conditions were adjusted to conform with those of the fractionated viruses.

Transcriptase assay. Fractionated viral samples in diluent solution were assayed for polymerase activity by mixing one volume of sample (either pellet alone, supernatant fluid alone, or pellet plus supernatant fluid) with one volume of a reaction mixture consisting of 6.5 $\times$ 10^{-4} M DTT, 8 $\times$ 10^{-3} M magnesium acetate, 5 $\times$ 10^{-2} M Tris-hydrochloride (*p*H 7.4), 1.4 $\times$ 10^{-3} M each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP), and 1.6 $\times$ 10^{-5} M ^{3}H-UTP (59 μCi/μmole). Samples (0.1 ml) were placed in individual test tubes, capped, and all except the zero time sample were incubated at 30 C. The reaction was terminated by the addition to each tube of 0.6 ml of 0.067 M sodium pyrophosphate and 0.1 ml (200 μg) of yeast RNA (2). The amount of ^{3}H-UTP incorporation was determined by precipitation of the protein and RNA with 0.5 ml of 25% trichloroacetic acid. The precipitates were collected on 0.45-μm membrane filters (Millipore Corp.) and washed with cold 5% trichloroacetic acid. The filters 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, and samples were counted for both ^{14}C-amino acid and ^{3}H-UTP label in a scintillation spectrometer. The zero time samples normally contained 100 to 200 ^{3}H counts/min, and this background was subtracted from the remaining times. ^{3}H incorporation was expressed relative to the ^{14}C internal protein marker so that all samples within an experiment could be normalized.

Polyacrylamide gel electrophoresis. The procedure for gel electrophoresis has been previously described (19); 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) were employed with a 0.1 M phosphate buffer (*p*H 7.4) also containing 0.1%

SDS. After electrophoresis for 5.5 hr, gels were fractionated into 1.25-mm slices. Protein was extracted from each slice during a 2-hr incubation at 50 C with 0.5 ml of Nuclear-Chicago solubilizer diluted with water (9:1). Ten milliliters of toluene-based scintillation fluid was added, and samples were counted in a Tri-Carb scintillation spectrometer.

RESULTS

Identification and purification of the protein or proteins responsible for the RNA-dependent RNA polymerase activity in VS virions would be greatly simplified if the polymerase enzyme could be solubilized. With this in mind, we fractionated VS virions and examined the separated and reconstituted fractions for their ability to incorporate ^{3}H-UTP into trichloroacetic acid-insoluble material. We chose to examine intact virions rather than the structurally simpler intracellular viral nucleocapsid for a number of reasons. First, intracellular nucleocapsids are likely to be contaminated with cellular enzymes or with hypothetical viral replicase. Secondly, analysis of unfractionated virions from the same preparation provides a convenient reference standard for quantitation of the amount of enzyme activity recovered in each fraction. Thirdly, the released virion can be isolated and purified as an intact unit which must possess all the proteins normally utilized in transcription. Because of the limited number of proteins in the virion (11, 17), each can be individually tested for any indirect, as well as direct, effect on transcription. Lastly, we wished to examine more thoroughly the transcriptase function in the T particle; reliable methods are available for partially purifying T as well as B virions (7).

Fractionation and reconstitution of template and enzyme of B virions. Nonionic detergents such as Triton N-101 have been used previously to prepare VS virions for polymerase assays (2, 5), but release of nucleocapsid cores from virions by the ionic detergent, deoxycholate, results in loss of polymerase activity (2). Triton X-100 which, in our hands, was as effective as Triton N-101 releases only the glycoprotein from the virion envelope in low-ionic-strength solutions (S. U. Emerson, *unpublished data*). However, we discovered that if the ionic strength is increased in the presence of Triton X-100, other viral proteins are solubilized. Experiments were designed to determine if the virion transcriptase was released from the template and if it retained activity under these conditions.

Fractionation of B virions suspended in RSB was accomplished by dilution with an equal volume of 2X Triton-high-salt solubilizer which contained Triton X-100, glycerol, DTT, and 1.44 M NaCl. After incubation for 1 hr at 30 C, the sample was transferred to a small cellulose nitrate tube and centrifuged at 125,000 $\times$ g for 90 min. Under these conditions the total virion ^{14}C-proteins were distributed about equally between a pellet and a supernatant fraction. The supernatant fraction was removed and diluted with 1.5 volumes of RSB to decrease the salt concentration; the pellet was resuspended in diluent solution made by adding high-salt solubilizer and 1.5 volumes of RSB. Portions of the supernatant and pellet fractions were recombined. Consequently, the pellet, supernatant, and mixed pellet-supernatant fractions were identical, except for viral constituents. Each sample was then diluted with an equal volume of polymerase assay mixture composed of magnesium acetate, DTT, and all four ribonucleoside triphosphates; 0.1-ml samples were incubated at 30 C to test for polymerase activity by incorporation of ^{3}H-UTP into trichloroacetic acid-precipitable material. The amount of virion protein in each reaction mixture varied with each experiment, but 1 to 2 μg of viral protein per 0.1 ml was generally used.

Figure 1 reveals that neither the supernatant fraction nor the pellet fraction contained significant polymerase activity when tested by itself. However, this procedure did not inactivate the polymerase because recombination of the pellet with the supernatant fraction resulted in excellent ^{3}H-UTP incorporation. Therefore, we could recover RNA polymerase activity from partially degraded virions from which at least 50% of the protein had been solubilized. This experiment also suggests that at least two components, one sedimentable and one soluble at 125,000 $\times$ g, are necessary for the polymerase reaction.

Some functional requirements of the reconstituted VS viral transcriptase system. In order to determine whether ^{3}H-UTP incorporation in the reconstituted pellet-supernatant system represents RNA production, single, unlabeled nucleoside triphosphates were removed from the assay mixture. Table 1 shows that omission of either ATP, CTP, or GTP completely eliminated ^{3}H-UTP incorporation (in agreement with previously reported observations of VSV transcriptase from unfractionated virions) (2).

Authentic transcription requires a minimum of two virion macromolecular components, a template, and a polymerase. An obvious question, therefore, is whether the Triton-high-salt solubilizer fractionates the virion so that one fraction contains the enzyme and the other contains the template. It could be argued that an unidentified coenzyme normally sequestered in the virion is released by the above treatment and is required for stimulation or activation of polymerase still

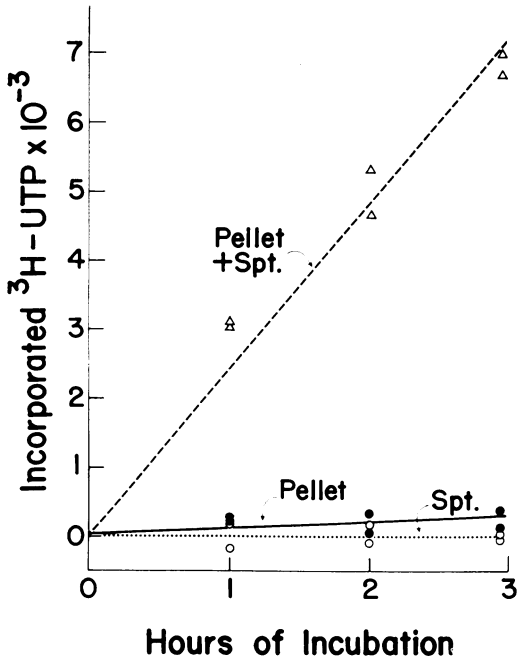

FIG. 1. *Polymerase activity of supernatant fraction (○), pellet (●), and reconstituted supernatant fraction plus pellet (△) of vesicular stomatitis virions fractionated by Triton-high-salt solubilizer. Purified B virions labeled with ^{14}C-amino acids were incubated in Triton-high-salt solubilizer, containing Triton X-100, glycerol, dithiothreitol, and 0.72 M NaCl, for 45 min at 30 C and then centrifuged in the SW50L rotor for 90 min at 125,000 × g. The pellet and supernatant fractions were separated and adjusted to the same chemical concentration by the addition of Triton-high-salt solubilizer plus RSB or RSB alone, respectively. In addition, equal volumes of diluted pellet and supernatant fractions were mixed, and this reconstituted system was also tested for transcriptase activity. Each sample (supernatant fraction, pellet, and a mixture of the two) was next mixed with standard polymerase assay mixture containing adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, ^{3}H-UTP, magnesium acetate, and dithiothreitol; 0.1-ml samples were distributed to capped tubes. After incubation at 30 C for 1, 2, or 3 hr, the polymerase reaction was terminated by adding to each tube 0.6 ml of 0.067 M sodium pyrophosphate and 0.1 ml (200 μg) of yeast RNA. The reaction mixtures were stored at −50 C and later thawed for assay. The RNA and virion proteins in each reaction tube were precipitated by adding 0.5 ml of 25% trichloroacetic acid and incubation at 0 C for at least 20 min. Precipitates were collected on 0.45-μm membrane filters; 0.3 ml of 0.3 N NaOH was then added to each filter to elute the precipitate. The ^{3}H-RNA and ^{14}C-protein radioactivity was counted by scintillation spectrometry. Background of 100 to 200 ^{3}H counts/min at zero time was subtracted from each time point. The amount of incorporated ^{3}H-uridine-5'-triphosphate was normalized to the ^{14}C-protein counts, which should be constant for each tube of a series to correct any errors due to losses in pipetting or filtering. Only minor corrections were necessary.*

TABLE 1. *Nucleoside triphosphate requirements of the vesicular stomatitis virion transcriptase activity reconstituted from the Triton-high-salt pellet and supernatant fractions*

Fraction[a]	Assay mixture	^{3}H-UTP incorporated (counts/min)[c]
Supernatant	Complete	188
Pellet	Complete	2,458
Supernatant and pellet	Complete	9,937
Supernatant and pellet	Minus adenosine triphosphate	0
Supernatant and pellet	Minus cytidine triphosphate	4
Supernatant and pellet	Minus guanosine triphosphate	25

[a] Purified VS virions labeled with ^{14}C-amino acids were separated into supernatant and pellet fractions by treatment with the Triton-0.72 M NaCl solubilizer as described in the legend to Fig. 1. Polymerase activity was tested in individual pellet and supernatant fractions and a reconstituted mixture of pellet and supernatant fractions.

[b] Conditions used for measuring the polymerase in complete assay mixture were the same as those described in the legend to Fig. 1. The complete and incomplete assay mixtures were identical except that one of three nucleoside triphosphates was omitted from the latter.

[c] Assay mixtures were incubated at 30 C for 3 hr before trichloroacetic acid precipitation. An average background radioactivity of 213 ^{3}H counts/min was subtracted. ^{3}H-UTP = ^{3}H-uridine-5'-triphosphate.

associated with the RNA template. However, heating a supernatant fraction or a pellet fraction to 100 C for 2 min completely abolished the ability of either to stimulate the incorporation of ^{3}H-UTP when mixed with an unheated complementary fraction. This experiment indicates that heat-labile macromolecules are involved in the activity of both fractions.

Additional studies revealed that transcriptase activity exhibits a quantitative dependence on the concentration of both the supernatant and the pellet fractions. In this experiment, pellet and supernatant fractions were prepared as before, by treatment of B virions with Triton-high-salt solubilizer, and centrifuged. Assays were performed by keeping the amount of pellet protein constant while adding decreasing serial concentrations of supernatant fluid and, conversely, by adding a constant amount of supernatant fraction to serially decreasing dilutions of a pellet fraction. As Fig. 2 demonstrates, there was a significant decrease in the amount of ^{3}H-UTP incorporated if

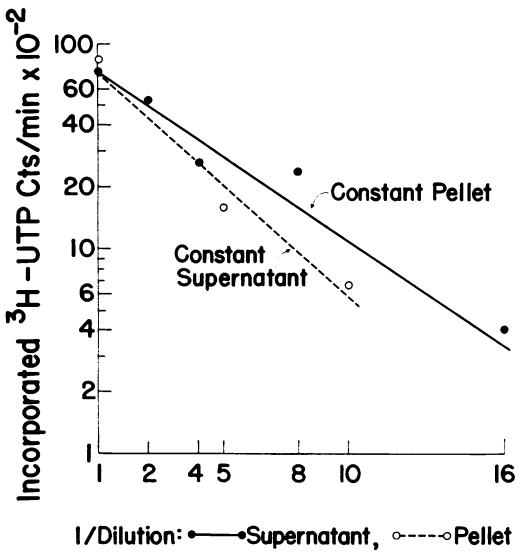

FIG. 2. *Reconstituted transcriptase activity as a function of concentration of pellet and supernatant fractions of vesicular stomatitis virions dissociated in Triton-high-salt solubilizer. Purified B virions were treated with Triton-high-salt solubilizer and separated into supernatant and pellet fractions as described in the legend to Fig. 1. Two different preparations were used. Undiluted samples of pellet or supernatant fractions were mixed with serially diluted samples of supernatant or pellet fractions in diluent solution. Mixtures of constant pellet with diluted supernatant fractions and constant supernatant with diluted pellet fractions were assayed for transcriptase activity by incorporation of ^{3}H-uridine-5'-triphosphate (^{3}H-UTP) into a trichloroacetic acid-insoluble fraction of a complete, standard polymerase assay mixture incubated at 30 C for 3 hr. The constant pellet fraction alone incorporated a background level of 2,458 counts/min of ^{3}H-UTP; the undiluted supernatant fraction had negligible transcriptase activity. The background ^{3}H-UTP incorporation of the constant supernatant fraction was 790 counts/min and that of the complementary pellet was 115 counts/min. These background radioactivities were subtracted from each mixed sample.*

either the pellet or supernatant fraction was diluted. The approximate exponential decline of transcriptase activity suggests enzyme kinetics.

The product of the reconstituted transcriptase system is complementary RNA. If transcriptase is active in the reconstituted system, the RNA product should hybridize with the complementary virion RNA. The RNA synthesized in a reconstituted system was mixed with virion RNA, and the extent of hybridization was measured as a function of resistance to ribonuclease digestion. Because transcribed molecules are released from the viral template upon completion (3, 4), a ^{3}H-UTP-incorporating reaction mixture containing reconstituted B supernatant and pellet was incubated at 30 C for 3 hr before centrifugation at 125,000 × *g* to separate the template from free, transcribed RNA. SDS (1%) was added to the supernatant fraction and ^{3}H-labeled RNA was extracted with phenol saturated with 0.01 M acetate buffer (*p*H 5.1). The RNA was ethanol precipitated and dissolved in 0.01 M Tris-HCl (*p*H 7.4) containing 0.4 M NaCl (1). Virion RNA was simultaneously extracted from unlabeled, purified B virions.

The ^{3}H-RNA transcription product (5,218 counts/min) was mixed with virion RNA at a concentration of 10 μg/ml, heated at 100 C for 30 sec, and then hybridized at 60 C for 1 hr (5); total reaction volume was 50 μliter. After a 30-min digestion with 10 μg each of pancreatic ribonuclease A and T_1 per ml, the samples were precipitated with trichloroacetic acid and counted. Controls either lacked added virion RNA or were not treated with ribonuclease. Only 10% (531 counts/min) of the RNA synthesized by the reconstituted enzyme system was ribonuclease resistant before hybridization. After hybridization with virion RNA, the ribonuclease resistance of the transcription product rose to 91% (4,727 counts/min), indicating complementarity. The transcriptase, therefore, constitutes the major enzyme activity in the reconstituted system.

Quantitative comparison of reconstituted and unfractionated virion transcriptase activity. The transcriptase is the only RNA polymerase activity that has been detected in VS virions so far (2, 4). However, it seemed important to prove that we were assaying the major polymerase activity in the virion rather than a minor side reaction. In other words, we needed to determine what percentage of the initial activity in the intact virion we were recovering by our fractionation procedure. To this end, the extent of ^{3}H-UTP incorporation in a reconstituted system was compared to that by unfractionated virions.

Three identical samples of purified B virions were mixed with an equal volume of either RSB alone, 2X Triton-high-salt solubilizer, or 2X Triton-low-salt solubilizer. Virions treated with RSB, which does not degrade the virus particles, served as an adequate control for loss of activity caused by inactivation of the polymerase during the incubation or centrifugation steps. Another control was to test a low-salt solution identical to the high-salt solubilizer, except that it contained only 60% as much NaCl. This served to determine whether the NaCl concentration rather than the glycerol or DTT was responsible for solubilization of virion proteins and polymerase activity. After incubation at 30 C for 1 hr in RSB, Triton-low-salt or Triton-high-salt solubilizers, 0.5 ml samples of VS-B virions labeled with ^{14}C-amino acids were partitioned by centrifuga-

tion at 125,000 × *g* into supernatant and pellet fractions. Samples of each were counted before and after centrifugation to determine the amount of ^{14}C-protein solubilized. All supernatant and pellet fractions, as well as recombined supernatants and pellets, were adjusted to the same ionic strength and chemical composition. Each supernatant and pellet fraction was assayed for polymerase activity alone, and in combination with its complementary fraction, and in one case the RSB pellet and high-salt supernatant fraction were recombined.

Table 2 and Fig. 3 show the detailed results of polymerase activity and ^{14}C-protein content of the supernatant, pellet, and recombined pellet-supernatant fractions of VS-B virions treated with RSB (Fig. 3A), Triton-low-salt solubilizer (Fig. 3B), Triton-high-salt solubilizer (Fig. 3C), and recombined RSB pellet and high-salt supernatant frac-

TABLE 2. *Comparative effect of solubilizers of different ionic strength on ^{14}C-protein and transcriptase activity dissociated from vesicular stomatitis virions and the degree of reconstitution of transcriptase activity by mixing the solubilized supernatant with the nucleocapsid pellet fractions*

Solvent[a]	^{14}C-protein solubilized (%)[b]	Transcriptase released (%)[c]	Supernatant fraction-enhanced transcriptase activity (%)[d]	
			Homologous pellet	RSB pellet
RSB	0	0	−7	
0.43 M NaCl-Triton	46.6	9	+10	
0.72 M NaCl-Triton	55.5	59.5	+50	−10

[a] Identical samples of vesicular stomatitis virions labeled with ^{14}C-amino acids were exposed to reticulocyte standard buffer (RSB), Triton-low-salt solubilizer, or Triton-high-salt solubilizer for 1 hr at 30 C. The radioactivity of each of the three samples before centrifugation was 65,000 counts/min of ^{14}C. A 0.5-ml amount of each sample was partitioned into supernatant and pellet fractions by centrifugation at 125,000 × *g* for 90 min.

[b] The percentage of protein solubilized = (^{14}C counts per minute per 10 μliters of supernatant fraction/^{14}C counts per minute per 10 μliters before centrifugation) × 100.

[c] Percentage of transcriptase released = 100% − (^{3}H-uridine-5′-triphosphate incorporated by 0.43 M or 0.72 M pellet)/^{3}H-UTP incorporated by RSB pellet × 100.

[d] Increased or decreased transcriptase activity of supernatant fraction added to homologous pellet or 0.72 M NaCl supernatant fraction added to RSB pellet compared with transcriptase activity of the RSB, 0.43 M NaCl, or 0.72 M NaCl pellet alone.

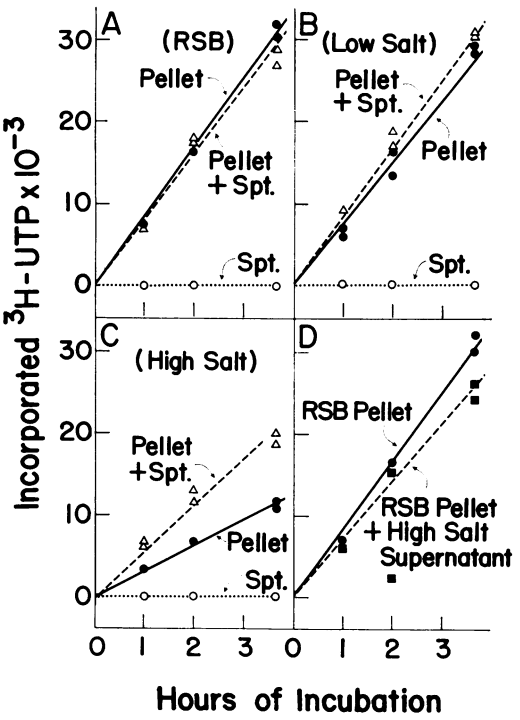

FIG. 3. *Quantitative dissociation of transcriptase activity with solubilizers of different ionic strength and reconstitution of transcriptional function of template and enzyme. A preparation of ^{14}C-amino acid-labeled B virions in reticulocyte standard buffer (RSB) was divided into three equal portions, each of which was diluted with one volume of RSB, 2X Triton-low-salt solubilizer (final concentration, 0.43 M NaCl) or 2X Triton-high-salt solubilizer (final concentration, 0.72 M NaCl). All samples were incubated at 30 C for 1 hr at which time the virus suspension was fractionated into pellet and supernatant fractions by centrifugation at 125,000 × g for 90 min. All samples were adjusted to the composition of diluent solution, and the pellet and supernatant fractions were assayed individually for transcriptase activity as were the recombined homologous pellets and supernatants of each preparation. In addition, the high-salt supernatant fraction was mixed with the RSB pellet to determine if ^{3}H-uridine-5′-triphosphate (^{3}H-UTP) incorporation could be further augmented by excess soluble enzyme (panel D). Transcriptase assays were performed in polymerase assay buffer containing all four nucleoside triphosphates and ^{3}H-UTP. All samples were normalized to ^{14}C-protein content so that all incorporation of trichloroacetic acid-precipitable ^{3}H-UTP could be directly compared on the same scale. Supernatant fraction (○), pellet (●), supernatant fraction plus pellet (△).*

tions (Fig. 3D). As noted, no protein or transcriptase activity was removed from VS virions in RSB; therefore, the transcriptase activity of these complete virions was considered to be 100%. On the other hand, low-salt buffer containing Triton X-100 solubilized 47% of the ^{14}C-protein, despite the fact that the pelleted virion cores retained 91% transcriptase activity (Table 2). Transcriptase activity was not significantly enhanced by recombining the pellet and supernatant fractions of either the RSB virions or the Triton-low-salt virions (Table 2 and Fig. 3A and B). In contrast, exposure to Triton-high-salt solubilizer removed 60% of the transcriptase activity along with 55% of the ^{14}C-protein from the pelleted VS virions. When the supernatant fraction solubilized by high-salt buffer was recombined with the high-salt pellet, the transcriptase activity of the pellet fraction was enhanced by 50%, equivalent to about 33% of the original RSB virion transcriptase activity (Fig. 3C and Table 2). Figure 3D demonstrates that this transcriptase activity in the high-salt supernatant fraction was unable to augment the transcriptase activity of the RSB pelleted, complete virions. This datum suggests that transcriptase activity of complete VS-B virions is optimal and cannot be enhanced by added transcriptase.

The data from these experiments support the hypothesis that liberation of a relatively small amount of virion protein reduces significantly the transcriptase activity of the virion, and this activity can be at least partially restored by recombination of the soluble protein and the virion core. The following experiments indicate that the major ribonucleoprotein N and viral RNA can together serve as template for the reconstituted transcriptase activity.

Distribution of virion proteins in the pellet and supernatant fractions. The data presented thus far are in agreement with the hypothesis that the virus is dissociated into a template and a polymerase fraction by Triton and high-salt treatment. It was of interest to determine which virion proteins remained in the template and which were solubilized. Virion proteins were labeled with ^{3}H-amino acids and the virions were purified and fractionated by incubation with Triton-high-salt and Triton-low-salt solubilizers and centrifugation. The proteins were trichloroacetic acid-precipitated to remove the salt and then washed with acetone to remove the trichloroacetic acid. Both the pellet and supernatant fractions were analyzed by electrophoresis on SDS polyacrylamide gels.

Figure 4 shows representative acrylamide gel profiles of the high-salt pellet and supernatant fractions. The high-salt supernatant fraction, which is necessary for restoring polymerase activity, contained proteins L, G, NS1, and M, but no N protein; the high-salt pellet fraction contained all of the nucleoprotein, a small amount of pro-

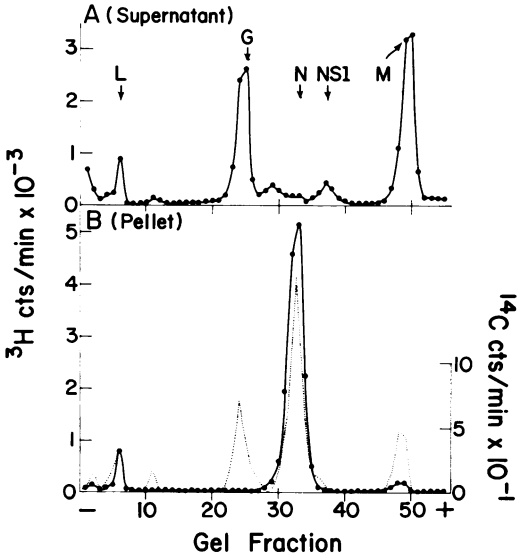

FIG. 4. *Electropherograms of ^{3}H-proteins (●) in the supernatant (A) and pellet (B) fractions of vesicular stomatitis (VS) virions dissociated by incubation in Triton-high-salt buffer. VS-B virions were grown on L cells in special Eagle basal medium lacking cold leucine and tyrosine but supplemented with ^{3}H-leucine and ^{3}H-tyrosine (5 μCi/ml each) to label viral proteins. Virions were purified as described in Materials and Methods by differential centrifugation and banding in 0 to 40% sucrose gradients. Purified B virions in reticulocyte standard buffer were diluted with one volume of 2X Triton-high-salt solubilizer to a final NaCl concentration of 0.72 M and fractionated by incubation and centrifugation as described in the legend to Fig. 1. The pellets were resuspended directly in 0.01 M N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 8 M urea, 1% mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) along with intact VS virions labeled with ^{14}C-amino acids added as marker. The supernatant proteins were also mixed with marker virions labeled with ^{14}C-amino acids and 50 μg of human hemoglobin added as carrier. Proteins were precipitated by addition of an equal volume of 20% trichloroacetic acid (TCA). The precipitates were collected by centrifugation at 1,000 × g and washed once with 10% TCA and twice with cold acetone (13). The precipitates were then dissolved in 0.01 M HEPES (pH 7.4) with 8 M urea, 1% mercaptoethanol, and 1% SDS. Both the pellet and supernatant samples were boiled for 2 min prior to electrophoresis on 7.5% SDS acrylamide gels; the gels were sliced and counted as described in Materials and Methods. The dotted lines show the pattern of ^{14}C-protein VS viral markers, and the arrows indicate the peak regions of viral proteins L, G, N, NS1, and M.*

tein L, and a trace of contaminating protein M. On the other hand, the low-salt supernatant fraction, which has little effect on RNA transcription, contained very little protein L, little or no NS1, but 90% of the G and M proteins (*data not shown*). Since the Triton-low-salt solubilizer releases virtually all of the G and M proteins and very little of the polymerase activity, G and M proteins are apparently not necessary for viral RNA transcription. The N protein was found exclusively in the high-salt pellet fraction and, therefore, cannot function alone as the transcriptase. Proteins L and NS1 were liberated with the polymerase activity by high-salt but not by low-salt solubilizer, and one or both must be required for enzyme function.

Additional evidence that the high-salt pellet is the nucleocapsid template. The preceding experiment demonstrated that the N protein is the major polypeptide of the high-salt pellet fraction, as is the case of the nucleocapsid liberated from VS virions by deoxycholate (6, 18). Further proof that the high-salt pellet is the virion nucleocapsid was sought by electron microscopy as well as by analysis of the RNA content of pellet and supernatant fractions.

The electron micrograph shown in Fig. 5 reveals that the high-speed pellet of VS virions fractionated by Triton-high-salt solubilizer was almost exclusively nucleocapsids (15, 18). No VS virions or other viral components could be detected in the many fields examined nor, as expected, could any cellular components. When purified VS virions labeled with ^{3}H-uridine were fractionated by Triton-high-salt solubilizer, 90% of the acid-precipitable ^{3}H label was present in the high-speed pellet. An attempt was also made to reconstitute transcriptase activity by adding enzyme in the VS virion high-salt supernatant fraction to templates of either deproteinized VS viral RNA extracted with phenol or a synthetic single-stranded polynucleotide. As reported for the whole virion by others (4), neither VS viral RNA nor the synthetic polynucleotide, poly G, was able to serve as template for the soluble transcriptase. These data suggest that the VS-B virion nucleocapsid, containing both N protein and RNA, is the essential template for the homologous transcriptase.

Reconstituted transcriptase activities of B and T virions. The above data demonstrate that we are analyzing a major portion of the transcriptase activity present in the VS virion. Because we can selectively solubilize certain of the virion proteins, including the polymerase, we can now reexamine the question of whether defective T particles contain the transcriptase. Purified T virions appear to lack polymerase activity, although qualitatively they contain the same proteins as B virions (5). Three possible explanations for lack of T-associated polymerase activity arise. (i) The polymerase molecules constitute such a small proportion of the total T virion proteins that the methods used to analyze the proteins have not been sensitive enough to detect the polymerase components; therefore, virion B may contain the polymerase enzyme, whereas T does not. (ii) Analysis of virion proteins by polyacrylamide gel electrophoresis cannot distinguish between active and inactive proteins; therefore, the T virion may have inactive polymerase proteins. (iii) The reaction we have studied requires at least two different sets of macromolecules, polymerase and template. T virions may contain potentially active polymerase, but the second component, the nucleocapsid-RNA template, may be defective. If T virions have an active transcriptase, it should be possible to solubilize the T enzyme and make it function by adding it to B template. Conversely, if T virions lack polymerase, it should be possible to isolate the supernatant fraction from normal B particles and test its ability to utilize T nucleocapsids as template.

Cells inoculated at a high multiplicity of infection produce both B and T virions, whereas those inoculated with a more dilute suspension of viruses produce predominately B particles (7). Because the B particles from a preparation containing large quantities of T are generally contaminated with the smaller T particle, separate L-cell cultures were inoculated at multiplicities of 0.5 plaque-forming unit/cell to produce predominantly B virions and at 25 plaque-forming units/cell to make T virions. The procedure used for ^{14}C-labeling, growing, and purifying the T particles was exactly the same as for B particles except that two bands were resolved on the 0 to 40% sucrose gradient, an upper one corresponding to fairly pure T (>90%) and a lower one containing both B and contaminating T; only the upper T band was collected. B virions were prepared simultaneously and collected from a separate sucrose gradient displaying only a B band virtually free of T particles. Because T virions produced by cultures infected at a high multiplicity always contain a certain number of B particles, even after separation in sucrose gradients (7), some criterion was required to demonstrate that this contamination of the T preparation with B virions was too low to obviate the experimental results. Therefore, plaque assays were performed to determine the number of B virions, and this figure was equated to the polymerase activity of a known concentration of pure B virions. The T

virion preparation had about 1% as many plaque-forming units per mg of viral protein as the B preparation, indicating a low level of contamination with B virions. When purified but unfractionated B and T particles were assayed for polymerase activity, the T preparation incorporated only 10% as much ^{3}H-UTP per mg of protein as did the B preparation. This amount of contaminating B particles in the T preparation was much too low to account for the following results. Equal volumes of B and T preparations adjusted to the same protein concentration were

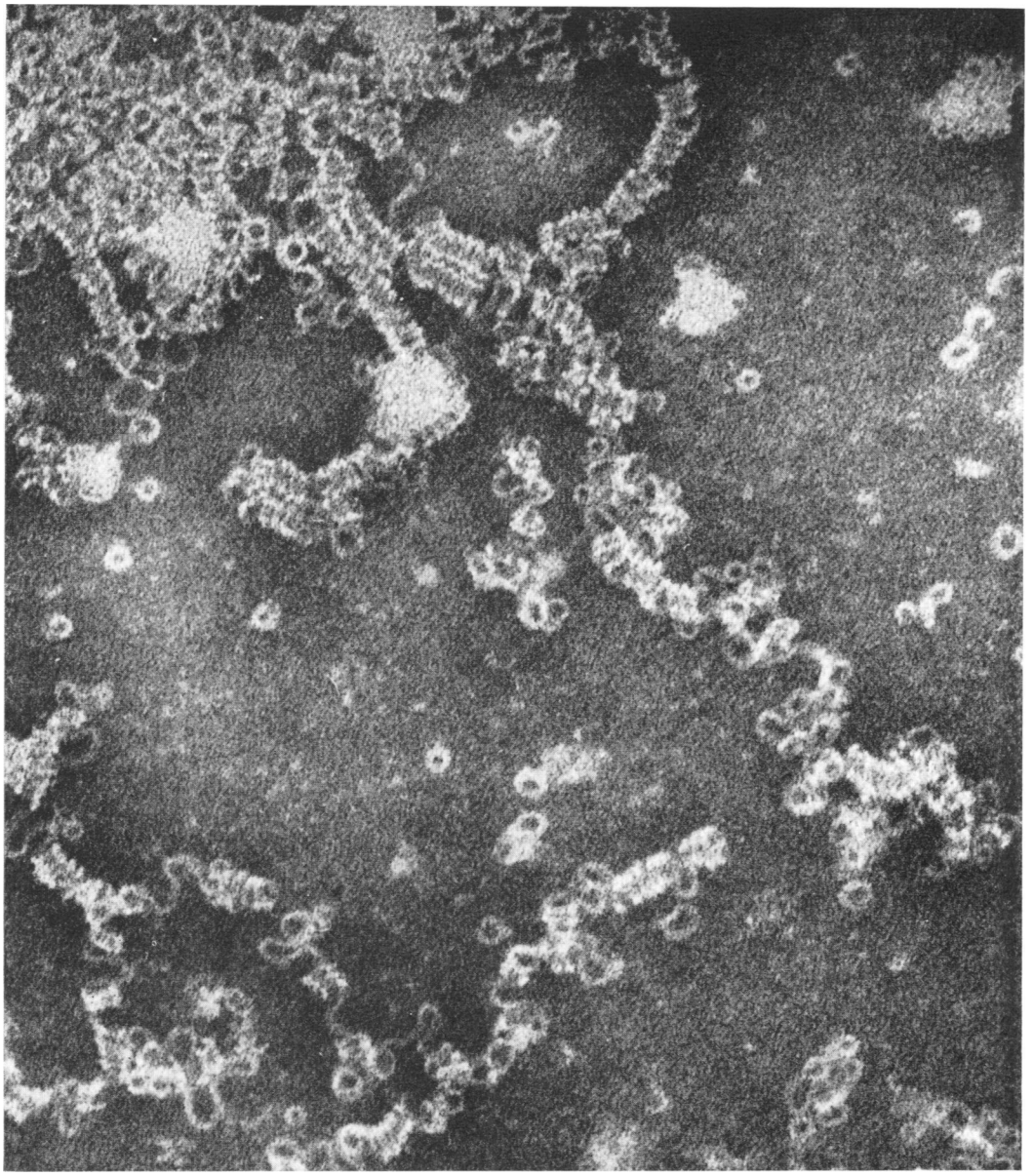

FIG. 5. *Demonstration that the pellet fraction of vesicular stomatitis B virions treated with Triton X-100 and 0.72 M NaCl consists of viral nucleocapsids. B virions were purified and fractionated in Triton-high-salt buffer as described in the legend to Fig. 4. The 125,000 × g pellet was resuspended in reticulocyte standard buffer, diluted with an equal volume of 2% phosphotungstic acid, and examined in a Siemens 1A electron microscope as previously described (18). No intact virions or other viral structures were observed. ×184,000.*

treated identically with Triton-high-salt solubilizer and fractionated by centrifugation, as previously described. SDS acrylamide gel analysis verified that T proteins were partitioned between the pellet and supernatant fractions the same way that B proteins were. Each supernatant fraction was then mixed with its homologous, pelleted template or a template prepared from the heterologous particles; these reconstituted fractions were assayed for polymerase activity by the standard procedure as was each individual fraction.

Figure 6 indicates that the B supernatant complements the B pellet as expected; the level of RNA synthesis in the homologous reconstituted B system was 17% that of the unfractionated B virus. The T template mixed with the T supernatant fraction produced very little RNA (about 8% that of the homologous, reconstituted B system); this RNA synthesis was approximately 15% of unfractionated T virions, the activity of which can be attributed to residual contaminating B virions. When a B supernatant fraction capable of complementing B template was mixed with the heterologous T template, virtually no RNA was made (Fig. 6C). Because this B supernatant fraction was effective in restoring transcriptase activity to a homologous B template, this result demonstrates that the T template cannot be transcribed in vitro by the B transcriptase. In the converse experiment, the T supernatant fraction which was inactive with T template was mixed with B template. In this case (Fig. 6C), as much or more RNA was synthesized on the B template in the heterologous combination as in the homologous B system.

Therefore, T virions contain the transcriptase enzyme which must account for approximately the same percentage of protein as in the B virion because the specific activities are quite close. Because T virions alone, or T nucleocapsid with T enzyme or B enzyme, do not demonstrate polymerase activity, the transcriptase found in the T virion clearly cannot utilize T nucleocapsid as a template.

Failure to demonstrate that T nucelocapsids compete with B template recombined with B or T transcriptase. The rationale behind these experiments was based on the often confirmed observation that defective T virions interfere in vivo with replication of infective B virions (10). This interfering activity appears to require T-RNA template function as demonstrated by marked sensitivity to ultraviolet light of T interfering activity (10). These findings have led to the postulate that a T-RNA template might compete with transcriptional or replicative functions of B polymerases (8, 16). The observations described in the preceding section, that T nucleocapsids were completely inactive as template for recombined B or T transcriptase, provided an ideal opportunity to test for the postulated competition of T nucleocapsids with B nucleocapsids for available soluble

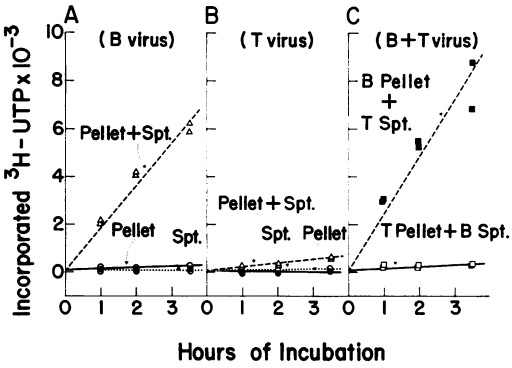

FIG. 6. *Demonstration that a polymerase dissociated from defective T virions can transcribe ³H-RNA on a nucleocapsid template isolated from B virions but not on a template isolated from the same T virions that yielded the polymerase. B and T virions were harvested from different cultures and purified separately by differential centrifugation and velocity sedimentation through 0 to 40% sucrose gradients, as previously described. The visible band in each gradient corresponding to B or T was collected, and the virions were pelleted and resuspended in reticulocyte standard buffer (RSB). The Lowry protein concentration of the B and T preparations was determined; then both were diluted with RSB to a final concentration of 750 μg of protein per ml. A 0.28-ml amount of each sample was mixed with 0.28 ml of 2X Triton-high-salt solubilizer to a final NaCl concentration of 0.72 M. The two samples were incubated at 30 C and fractionated as shown in Fig. 1 by centrifugation at 125,000 × g. The supernatant and pellet fractions were diluted identically with RSB or diluent solution so that each fraction of T had the same volume and chemical composition as that of B. Polymerase activity was determined as shown in Fig. 1. Both the unmixed B and T supernatant fractions and pellets were assayed alone. Each supernatant fraction was also mixed with the B template and, separately, with the T template; transcriptase activity was assayed by ³H-uridine-5'-triphosphate incorporation. Because the protein concentrations were the same in the B and T preparations prior to their identical fractionation, the B and T fractions are equivalent and quantitative comparisons can be made directly. A, The homologous B virion system showing the polymerase activity of the B supernatant fraction (○), B pellet (●), and the reconstituted B supernatant fraction plus B pellet (△). B, The corresponding homologous polymerase assay system for T particles showing the T supernatant fraction (○), T pellet (●), and T supernatant fraction plus T pellet (△). C, The heterologous combination of T pellet assayed for polymerase activity with the B supernatant fraction (□) and B pellet mixed with the T supernatant fraction (■).*

transcriptase. If T virions can interfere with transcription on a B template, the presence of T nucleocapsids in a reconstituted system with B template and enzyme should result in a decrease in RNA synthesis in vitro, due to competition for the transcriptase.

Figure 7 shows that the presence of T nucleocapsids had no effect on ^{3}H-UTP incorporation when B template was reconstituted with either B- or T-soluble polymerase. The absence of T interference was noted despite the fact that the experimental reaction mixtures contained about three times as many T-RNA molecules as B-RNA molecules.

For this experiment to be valid, the transcriptase should be limiting. That polymerase was not saturating is indicated in a number of ways. First, the amount of supernatant protein added back to the pellet is less than was originally removed. Secondly, incorporation by the homologous B system is only 17% that of the unfractionated virion, whereas in Fig. 3 we demonstrated that recovery of at least 33% was possible. In addition, the ratio of B template to supernatant fraction in the competition experiment presented in Fig. 7 was half that depicted in Fig. 6. Because the protein concentrations used for the experiments shown in Fig. 6 and 7 were normalized to each other, the amounts of incorporation can be compared directly. As can be seen, decreasing the amount of B pellet by a factor of 50% while keeping the amount of supernatant fraction constant decreased the total incorporation by only 24%, indicating that the template is in excess (see also Fig. 2). However, addition of T template to a mixture of B template and transcriptase from either T or B particles had no significant effect on ^{3}H-UTP incorporation. Therefore, T nucleocapsids must not effectively compete with B template for the transcriptase molecule.

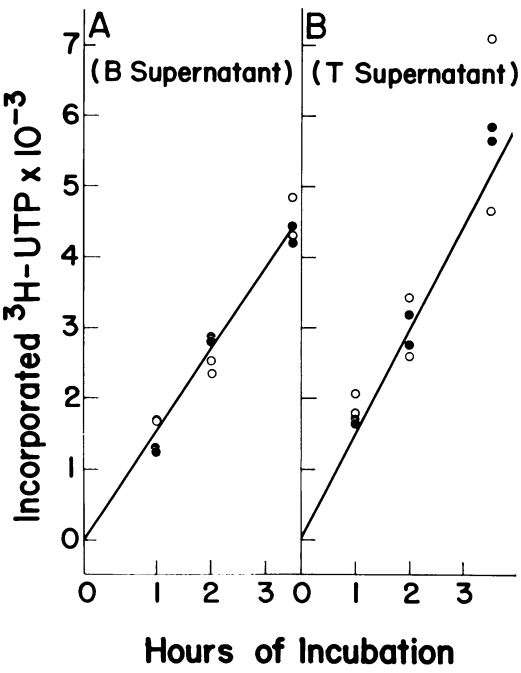

FIG. 7. *Inability of T nucleocapsids to effect the template activity of B nucleocapsids recombined with soluble transcriptase from B and T virions. Samples of the same B and T pellets and supernatant fractions shown in Fig. 6 were recombined and assayed for polymerase activity with some modifications. The assay mixtures contained half the concentrations of B (or T) pellet but the supernatant concentration was unchanged. Therefore, the ratio of supernatant fraction to B pellet was twice that shown in Fig. 6. Two of the four samples contained a mixture of B and T pellets plus supernatant fractions of either B or T; the other two tubes contained only B pellet and B or T supernatant fractions plus diluent to compensate for volume. Because the B and T nucleocapsids were prepared from the same protein concentration of virions, the absolute amount of RNA present in the B and T nucleocapsids should be identical; however, the molar ratio of T RNA to B RNA should be about 3 to 1, reflecting the threefold greater molecular weight of B RNA. A, B supernatant mixed with B template alone (○) or with B template plus T template (●). B, T supernatant mixed with B template alone (○) or with B template plus T template (●).*

DISCUSSION

An RNA-dependent RNA polymerase which transcribes RNA complementary to the viral genome has been conclusively demonstrated in Triton-treated VS-B virions; the chemical requirements for this enzyme activity have been well documented (2, 4). In these original experiments, however, the virion retained a large degree of structural integrity for the only protein solubilized is the spike protein G. Analysis of the molecular requirements for transcription requires that a functionally active enzyme be isolated free of template. With this tool in hand, precise questions regarding effects, such as the action of interferon-induced products on viral transcription or the T-mediated interference of VS-B replication, can be asked by using cell-free systems. The obvious disadvantage of transcriptional studies with the undissociated template-enzyme system of intact VS virions is that competing inhibitors might not work unless enzyme action requires reinitiation.

Do all in vivo transcriptional events occur in vitro? The hybridization data of Bishop and Roy (5) indicate that some of the product RNA synthesized by Triton-treated VS virions is in molec-

ular excess relative to input template RNA and, therefore, must arise from repetitive transcription of part of the genome. Repetitive transcription in this system could reflect termination of transcription followed by reinitiation by the same polymerase molecules. However, repetitively transcribed sequences could also be produced if the viral genome were packaged with multiple copies of polymerase attached at more than one initiation site; sequential transcription, under this condition, could produce the same net RNA products as reinitiation, without the requirement for de novo attachment of polymerase to template as is the case in reinitiation. There is presently no simple way to distinguish between these two possibilities in this particular system. The Triton-high-salt fractionation and reconstitution experiments, however, demonstrate that binding of polymerase and initiation of transcription can occur in vitro.

The interpretation most consistent with our results is that the virion transcriptase system has been fractionated into two components, a soluble enzyme and an insoluble nucleocapsid template composed of both RNA and N protein. Since the nucleocapsid and supernatant fractions can be prepared virtually free of UTP-incorporating activity (see Fig. 6A), it is highly unlikely that either fraction is acting simply to enhance a low level of endogenous activity present in the other fraction. In other words, the supernatant fraction must be supplying all or part of the polymerase molecule itself. If the transcriptase is solubilized by Triton-high-salt solubilizer, RNA synthesis can presumably occur in the reconstituted system only if the enzyme rebinds at a site on the template where transcription can be initiated. The reconstitution of enzyme activity should provide an excellent model system for examination of initiation and all other parameters affecting viral RNA transcription.

T particles interfere with B particle production in vivo, possibly by competitively inhibiting either transcription or replication of the B genome (7, 12, 16). By employing the Triton-high-salt fractionation and reconstitution system, we have demonstrated that the T nucleocapsid does not interfere with transcription of the B genome in vitro. The B and T nucleocapsids were mixed together prior to the addition of supernatant fractions; therefore, we interpret the lack of inhibition by T nucleocapsid to mean that polymerase in the supernatant reattached preferentially to the B nucleocapsid, even though there were approximately three times as many T nucleocapsids present. These results suggest that the transcriptase has a lower affinity for the T nucleocapsid or does not bind to the T-particle ribonucleocapsid at all. Additional experiments are in progress to determine if the transcriptase is ever attached to ribonucleoprotein from T particles. Although it is difficult to interpret in vivo observations on the basis of in vitro results, the B and T mixing experiments suggest that it is very unlikely that the T particle interferes with B particle production at the level of transcription, a result consistent with the in vivo observation of Huang and Manders (8).

Initiation of transcription upon reconstitution of B virion pellet and supernatant fractions permitted reexamination of the question of template specificity. In previous experiments with Triton-treated virions, exogenously added viral RNA or synthetic polynucleotides did not appear to serve as templates for transcription (4). These experiments are difficult to interpret because it is not clear whether polymerase was ever released from the original template to be made available for exogenous template in the same reaction mixture. Our extraction of transcriptase by Triton-high-salt solubilizer provides an important means of approaching the question of template requirements of the reactivatable enzyme completely freed of its natural template. However, even when soluble polymerase was presented with nonnucleocapsid RNA species as possible templates, no transcription occurred. Supernatant enzyme, which stimulated RNA production when added to B nucleocapsid, was unable to synthesize RNA when virion RNA deproteinized by phenol extraction or polyguanylic acid was substituted. This result indicates that stringent template requirements must be met before transcription can proceed and suggests that the N protein may be necessary. However, not just any viral N protein-RNA complex is sufficient to serve as template for transcription. This was clearly demonstrated by the observation that the T nucleocapsid could not serve as a template for transcriptase active on B nucleocapsids. If one assumes that the N protein is identical in both B and T particles, the T RNA itself must be defective, and the VS virion transcriptase must require not only N protein, but a specific RNA sequence for initiation of enzyme activity.

The exact polypeptides which comprise the actual polymerase molecule have not yet been identified. Triton-low-salt solubilizer removes 90% or more of both the G and M proteins from the nucleocapsid cores without significantly decreasing RNA synthesis. Therefore, neither G nor M protein is required for transcription, and neither affects the amount of RNA produced, although it is still possible that one or both proteins could affect the fidelity of the RNA product. Because the majority of G and M protein mole-

cules can be removed without affecting the rate of RNA synthesis, one or more of the minor proteins of the virion must constitute at least part of the active polymerase. An enzymatically active nucleocapsid preparation contains L, NS1, and N proteins and possibly other proteins present in quantities too small to be detected. It seems unlikely that N protein, which is present in a much greater molar ratio than the other nucleocapsid proteins, could interact stoichiometrically with any of these molecules to form an active complex. Unless deproteinized RNA can be utilized as template, it will be difficult to prove that N protein is not an integral part of the polymerase molecule, but such direct participation of N protein appears unlikely on a priori grounds. In preliminary experiments, recombination of a Triton-high-salt pellet and supernatant fraction resulted in isolation of the transcriptase reactivated nucleocapsid that reassociated with both L and NS1, but not G or M proteins. Experiments now in progress should clarify which of the two minor VS nucleocapsid proteins are necessary for transcriptase function.

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