Isolation of an Infectious Ribonucleoprotein from Vesicular Stomatitis Virus Containing an Active RNA Transcriptase

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A ribonucleoprotein complex (TNP) containing an active RNA polymerase was isolated from purified vesicular stomatitis virus particles. The TNP sedimented through a sucrose gradient as a single band and appeared under the electron microscope as discrete long filaments in a spiral configuration. TNP contained one major and two minor polypeptides, but not the polypeptides associated with the outer coat of vesicular stomatitis virus. BHK-21 clone 13 cells could be infected with TNP, yielding infectious virus particles.

Vesicular stomatitis virus (VSV) contains a single-stranded RNA molecule (9, 14) and four or five polypeptides (10, 14, 17, 18). The virions contain an RNA-dependent RNA transcriptase, and virus particles which have been treated with a neutral detergent can then in vitro synthesize RNA molecules with base sequences complementary to the base sequence of virion RNA (3, 4). Polysomes isolated from infected cells contain single-stranded RNA molecules with nucleotide sequences complementary to virion RNA (8, 15). It has been suggested that it is not the virion RNA, but rather a nucleoprotein complex containing the RNA polymerase, which is essential for infection of the cells (1, 2). After this complex has entered the cell, the transcriptase synthesizes the messenger RNA molecules necessary for further virus development.

When virus particles were disrupted by the addition of a neutral detergent and centrifuged through a sucrose gradient, the particulate fraction had RNA transcriptase activity, indicating close association of at least some of the RNA transcriptase with the virion RNA (J. F. Szilágyi and C. R. Pringle, in press).

Here we describe the purification from VSV particles of a ribonucleoprotein complex (TNP) with RNA transcriptase activity from which the proteins associated with the outer coat of the virus have been removed by the combined action of a neutral detergent and CsCl.

MATERIALS AND METHODS

Virus growth and purification. VSV, Indiana serotype, was grown in BHK-21 clone 13 cells (13) in rotating 2.5-liter bottles infected with purified virus suspension at an approximate multiplicity of 0.01 plaque-forming units (PFU)/cell. The culture medium (40 ml of Eagle medium containing 4% calf serum) was removed after 16 to 24 hr of incubation at 31 C, and the cell debris was discarded after centrifugation at 3,000 rpm for 30 min. The virus was subsequently sedimented by centrifugation at 21,000 rpm for 90 min (MSE 65, 10 x 100 ml rotor). The combined pellets from three bottles were suspended gently in 5 ml of buffer (containing 20 mM tris (hydroxymethyl) aminomethane [Tris]-hydrochloride buffer, pH 8.0, 0.1 mM NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA]) and centrifuged through a linear 15 to 40% sucrose gradient (made up in the same buffer) at 21,000 rpm for 90 min (MSE 65, 3 x 65SW rotor). Pasteur pipettes with upturned tips were used to collect the virion zone, and the sucrose was removed subsequently by dialysis against 20 mM Tris-hydrochloride buffer, pH 8.0. The protein was determined by the method of Lowry et al. (12) with bovine serum albumin as standard.

H-labeled virus was prepared as described above by adding [5-3H]uridine (200 μCi for each bottle, Radiochemical Centre, Amersham) to the medium 6 hr after infection. 3H-labeled virus was obtained in a similar way, except that the Eagle medium used contained only 10% of its usual amino acid concentration and 1-methionine-S35 (200 μCi for each bottle, Radiochemical Centre, Amersham) was introduced 6 hr after infection. 3H-labeled T particles were obtained in a manner similar to that for 3H-la-
beled B virus particles, except that the multiplicity of infection was 5 PFU/cell.

**Disruption of virus and purification of TNP.**

Virus particles were disrupted by mixing 40 μl of virus suspension containing 400 μg of virus protein with (final concentrations): 20 mM Tris-hydrochloride buffer (pH 8.0), 5% glycerol, 5 mM EDTA, 3.5 mM dithiothreitol, 0.1% N101, and 0.5 mM CsCl, in this order, in a final volume of 0.6 ml. After standing at 0 C for 20 min, 0.6 ml of 20 mM Tris-hydrochloride buffer (pH 8.0), containing 3.5 mM dithiothreitol, was added.

This suspension of disrupted virus particles (1.2 ml) was placed on a linear 15 to 40% sucrose gradient (made up in buffer containing 20 mM Tris-hydrochloride buffer [pH 8.0], 0.1 mM NaCl, 1 mM EDTA, and 3.5 mM dithiothreitol and layered over a 1-ml cushion of 60% sucrose), centrifuged at 21,000 rpm for 220 min at 5 C (MSE 65, 6 × 15 SW rotor), and 6-drop fractions were collected.

In other experiments, the suspension of disrupted virus particles was placed on a linear 25 to 45% glycerol gradient containing 20 mM Tris-hydrochloride buffer (pH 8.0), 0.1 mM NaCl, 1 mM EDTA, and 3.5 mM dithiothreitol and centrifuged at 45,000 rpm for 100 min at 5 C (Spinco SW65 head). Then the soluble proteins from the top of the gradient were removed by cutting the centrifuge tubes one-third from the bottom. The pelleted TNP was suspended in 40 uliters of 20 mM Tris-hydrochloride buffer (pH 8.0) so as to correspond to the volume of virus suspension from which it was obtained.

**RESULTS**

**Purity of the virus.** The purified virus particles, when centrifuged through a sucrose gradient, produced a single sharp band (peak at fraction 17; Fig. 1a); no peak corresponding to T particles (peak at fraction 22; Fig. 1b) was observed. Therefore the purified virus particles were uniform in size and almost completely free of T particles.

**Properties of the TNP.** The originally turbid virus suspension became almost water-clear when treated with 0.1% triton N101 and 0.5 mM CsCl, indicating solubilization of some of the proteins and configurational changes in the ribonucleoprotein core.

After such disruption of the virus particles and subsequent centrifugation through a su-

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**Fig. 1.** Sedimentation characteristics of [3H]uridine-labeled virus, Suspensions of purified virus particles (a) and T particles (b), both labeled with [3H]uridine, were diluted in a solution containing (final concentration): 20 mM Tris-hydrochloride buffer (pH 8.0), 0.1 mM NaCl, 1 mM EDTA, and 3.5 mM dithiothreitol. The diluted suspensions were centrifuged at 21,000 rpm for 100 min at 5 C (MSE 65, 6 × 15 SW rotor) through a linear 15 to 40% sucrose gradient layered over a 1-ml 60% sucrose cushion, both containing 20 mM Tris-hydrochloride buffer (pH 8.0), 0.1 mM NaCl, 1 mM EDTA, and 3.5 mM dithiothreitol, and 5-drop fractions were collected. From each fraction, a 50-μlter amount was placed on Whatman no. 1 filter-paper discs and washed three times in 5% trichloroacetic acid containing 40 mM sodium pyrophosphate, twice in ethanol, and once in ether. Radioactivity was measured by liquid scintillation spectrophotometry.
crose gradient, a single peak slightly below the middle of the gradient (peak at fraction 13) was detected when \( ^{3}H \)uridine-labeled virus was used (Fig. 2a). The sedimentation rate of this material indicated that some protein was still associated with the RNA. The sharpness of this peak indicated that most of the complex was uniform in size and configuration. No labeled virus was found on the 60% sucrose cushion at the bottom of the gradient (fractions 2 to 4), showing that the disruption of the virus particles was complete. There was a small amount of labeled material on the top of the gradient, probably free RNA. No peak corresponding to the ribonucleoprotein of T particles (approximate position would be in fractions 20 to 25) was observed.

When \( ^{35}S \)methionine-labeled virus was used, two peaks were detected in the gradient (Fig. 2b). One corresponded with the \( ^{3}H \)-uridine-labeled peak whereas the other was at the top of the gradient. Thus the peak in the middle of the gradient contained ribonucleoprotein and the solubilized proteins remained on the top of the gradient.

Assay of the RNA transcriptase activity in each fraction of the gradient showed that the ribonucleoprotein peak contained RNA tran-

![Fig. 2. Characterization of TNP by sucrose gradient centrifugation. The virus particles were disrupted and centrifuged through a 15 to 40% sucrose gradient as described. a. Virus labeled with \( ^{3}H \)uridine; b, virus labeled with \( ^{35}S \)methionine. The radioactivity in 50 μl of each fraction was measured as described in the legend to Fig. 1. c, RNA transcriptase activity in 0.1 ml of each fraction of gradient a was assayed for 60 min at 31 °C as described in the legend to Fig. 5, except that NaCl was omitted from the reaction mixtures. d, Infectivity in each fraction of gradient a was titrated in BHK-21 clone 13 cell monolayers using solution b (see Table) for the dilution of the fractions.](http://jvi.asm.org/Downloadedfrom)
scriptase activity (Fig. 2c). For this reason we called this ribonucleoprotein preparation "transcribing nucleoprotein" or "TNP."

Titration of the fractions in BHK-21 clone 13 cells showed that all infectivity was associated with the TNP peak (Fig. 2d). Thus the TNP both contained an active RNA transcriptase and had retained the potential to produce infectious virus particles. The successful disruption of all the virus particles was confirmed by the complete lack of infectivity of material on the surface of the 60% sucrose cushion.

**Structure and composition of TNP.**

When [3H]uridine-labeled virus was used, 100% of the radioactivity was recovered in the TNP which was purified by pelleting it through a glycerol gradient and, when [35S]methionine-labeled virus was used, about 50% of the radioactivity was recovered. Thus the TNP contained all the virion RNA and about half of its protein.

The electron microscope disclosed the TNP as discrete long filaments in a spiral configuration, appearing (Fig. 3) either as extended spirals (S) or loose conglomerates (C).

Polyacrylamide gel electrophoresis of the TNP showed that it contained three polypeptides of the virus particles, the nuclear (N) polypeptide, the large (L) polypeptide, and the so-called nonstructural (NS) polypeptide (Fig. 4, gel 2).

The solubilized proteins on the top of the gradient contained the two polypeptides associated with the coat of the virus, the glycopolypeptide (G) and the matrix (M) polypeptide (Fig. 4, gel 3). Another (A) polypeptide was also detectable in this fraction. This polypeptide was found in small quantities between the polypeptides G and N, and in some experiments it was resolved into two separate bands (Fig. 4, gel 1). The solubilized proteins did not contain detectable amounts of the three polypeptides associated with the TNP, except for traces of the N polypeptide. Densitometer tracing of the radioautographs showed that the purified virus contained approximately 54% polypeptide N, 24% polypeptide M, 15% polypeptide G, 4% polypeptide L, 2% polypeptide NS, and only a trace of A. Similar tracings of the radioautograph of TNP showed that it contained the polypeptides N, L and NS in the same ratios as in the virus particles.

The TNP was partially resistant to ribonuclease since, after incubation at 37°C for 1 hr in the presence of 10 or 50 μg of pancreatic ribonuclease per ml, approximately 85 or 60%, respectively, of the [3H]uridine label in the RNA component of the TNP remained in acid-insoluble form.

**Characterization of RNA synthesis by TNP.**

The in vitro RNA synthesis at 31°C by the resuspended TNP continued for at least 180 min, although it was linear only for the first 45 min (Fig. 5a). The amount of RNA synthesized during this first 45 min slightly exceeded that synthesized by an equivalent amount of virus particles during the same time. Thus most of the RNA transcriptase molecules were retained by the TNP. The RNA synthesis by TNP continued throughout the experiment, whereas a plateau was reached after 120 to 150 min during RNA synthesis by the virus particles. This could be explained by the removal during centrifugation of some solubilized substance which interferes with the transcription process. The RNA transcriptase activity of TNP is heat-labile: preincubation of the TNP at 31°C for 1 hr before the
addition of the other ingredients of the reaction mixture resulted in about a 55% loss of the enzyme activity, whereas similar preincubation at 39 C led to total loss of enzyme activity (Fig. 5b). When the TNP was preincubated at 31 C in the presence of all ingredients of the reaction mixture except MgCl₂, the transcriptase activity was only slightly affected, and after preincubation at 39 C about 52% was still retained (Fig. 5c). Dithiothreitol contributed to this increase in heat stability, since 76% of the enzyme activity was retained after preincubation at 31 C and, even after preincubation at 39 C a trace of residual enzyme activity was detectable (Fig. 5d). But Fig. 5e shows that the combined effect of the other ingredients of the reaction mixture provided most of the protection. The heat-sensitive component of the TNP was not identified.

Titration of TNP infectivity. Characteristics of the infectivity of the TNP are summarized in Table 1 part A. When the TNP was diluted in phosphate-buffered saline containing 0.5% calf serum (solution a), its infectivity was about 7 log units less than that of an equivalent amount of virus (3.4 and 10.2, respectively).

The addition of diethylaminoethyl-dextran, dithiothreitol, and glycerol to solution a (solution b) increased the infectivity of the TNP by 3 log units (from 3.4 to 6.2) whereas that of the virus particles was affected only slightly (an increase from 10.2 to 10.5). The greatest increase in the infectivity of TNP was obtained when all three compounds were added to solution a. Added singly, diethylaminoethyl-dextran (solution c) had the most marked effect, an increase from 3.4 to 5.6 log units. Dithiothreitol alone (solution d) also increased infectivity (from 3.4 to 4.3), and even the addition of glycerol had a slight effect (from 3.4 to 3.9).

The infectivity of the TNP was severely reduced (from 5.8 to 3.6) by ribonuclease and after treatment with chymotrypsin no residual infectivity of TNP was detected (Table 1, part B).

**DISCUSSION**

From virus particles disrupted with a neutral detergent and CsCl a ribonucleoprotein complex (TNP) can be isolated by density-gradient centrifugation.

The sedimentation of the TNP in a sharp band through the sucrose gradient indicated that its configuration was uniform. However,
under the electron microscope, the filaments of the pelleted TNP appeared not only in the form of extended spirals but also as loose conglomerates. This lack of uniformity in the resuspended TNP is presumably a result of the pelleting. The TNP filaments were similar in appearance and presumably identical to the extended strands which were obtained by Nakai and Howatson after storage in the cold of purified VSV particles (16).

The TNP contained three polypeptides (L, N, and NS) of the virus, whereas the solubilized proteins at the top of the gradient contained the three other polypeptides of the virus (G, M, and A) and traces of N. Thus all the coat proteins were solubilized by our method, whereas the polypeptides of the core remained almost entirely in close association with RNA. This is corroborated by the densitometer tracing which showed that N, NS, and L comprised approximately 60% of the virus protein, which corresponded reasonably to the 50% of virus protein recovered from the pelleted TNP. This leads us to believe that not only N but also L and NS are part of the virus core.

The RNA transcriptase activity in the TNP was greater than that in an equivalent amount

![Graph](http://jvi.asm.org/)  
**Fig. 5.** Characteristics of the RNA transcriptase of TNP. a, One reaction mixture (0.4 ml) contained 0.05 ml of resuspended TNP (■) and the other, 0.05 ml of virus suspension (□); both were incubated at 31 C. In b, c, d, and e, 0.05-ml suspensions of TNP were incubated at 0 C (●) at 31 C (○) and at 39 C (△) for 1 hr before enzyme assay. During the preincubations, nothing was added to the TNP in b; in c all the ingredients of the reaction mixture except MgCl₂ were present. In d, 3.5 mM dithiothreitol was added to the TNP, and in e all ingredients of the reaction mixture except dithiothreitol and MgCl₂ were added. The suspensions were then cooled to 0 C, the remaining ingredients were added, and the residual RNA transcriptase activities were assayed at 31 C by a slightly modified method of Baltimore et al. (3). The reaction mixtures (0.4 ml) contained either 0.05 ml of virus or resuspended TNP; 3.5 mM dithiothreitol; 0.1 M Tris-hydrochloride (pH 8.0); 0.1 M NaCl; actinomycin D (16 µg/ml); 0.64 mM each adenosine 5'-triphosphate, cytidine 5'-triphosphate, and guanosine 5'-triphosphate; 0.64 mM uridine 5'-triphosphate, and uridine [5-³H]triphosphate (specific activity 15 Ci/mmole, Radiochemical Centre, Amersham, in 50% ethanol evaporated before use). The reaction mixture contained approximately 25,000 counts/min in 20 µlitters. To those reactions mixtures which contained the virus, triton N101 (0.04%) was also added. The reaction was started by the addition of 5 µlitters of 440 mM MgCl₂ after 1 min of warming up to the incubation temperature (31 C). Samples (20 µlitters) taken both before the addition of MgCl₂ (0 time) and during incubation were withdrawn and placed on Whatman no. 1 filter-paper discs, washed seven times in ice-cold 5% trichloracetic acid containing 40 mM sodium pyrophosphate, twice in ethanol, twice in ether, and then dried; radioactivity was measured by liquid scintillation spectrophotometry.
of virus particles, especially in the later stages of incubation. Thus transcriptase is strongly associated with the viral RNA, since no detectable loss of enzyme activity occurred during the disruption of the virus particles and the subsequent isolation of the TNP. The higher rate of enzyme activity in the later stages of in vitro RNA synthesis could be explained by the removal of either an enzyme inhibitor or of a ribonucleoprotein originally on or in the virus particles.

Infectious virus particles were produced after the infection of animal cells by TNP. Two separate experiments showed that undisrupted virus particles could not have been responsible for the infectivity of the TNP preparation. First, all infectivity was associated with the TNP peak in the sucrose gradient, whereas none was observed on the surface of the 60% sucrose cushion where intact virus particles would be found. Second, the infectivity of the resuspended TNP was increased after the addition of diethylaminoethyl-dextran, which protects the TNP from ribonuclease, and dithiothreitol, which increased its heat stability. These findings are in good agreement with the findings of Brown et al. (6), who treated the virus with Tween 80 and ether and obtained a subviral structure whose infectivity was not neutralized by virus-immune serum. However, their subviral structure retained the overall shape of the virion, whereas the TNP was in the form of loose spirals. The production of infectious virus particles by TNP seems to confirm the hypothesis that a ribonucleoprotein complex containing an active RNA transcriptase is essential for infection of the cells.

Since this work was completed, an article by Emerson and Wagner appeared in which they described the disruption of the virion by the combined action of a neutral detergent and a salt (7). With a high concentration of NaCl, they separated the RNA transcriptase activity from the ribonucleoprotein whereas, at a lower salt concentration, the enzyme activity remained attached to the ribonucleoprotein. Our findings and those which they obtained after the triton low-salt treatment are essentially in agreement. Our findings are also in agreement with those of Bishop and Roy (5).

We are now investigating whether one of the polypeptides or a combination of any of the three polypeptides of the TNP is responsible for the RNA transcriptase activity.

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**LITERATURE CITED**

2. Baltimore, D., A. S. Huang, K. F. Manly, D. Rekosh,


