Function and Structure of RNA Polymerase from Vesicular Stomatitis Virus*

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The RNA-dependent RNA polymerase associated with vesicular stomatitis virus was isolated to apparent homogeneity by a newly developed procedure, which includes stepwise removal of proteins from virions by successive treatments with high concentrations of cesium sulfate and cesium chloride, followed by glycerol gradient centrifugation or chromatography on phosphocellulose or DEAE-Sephadex column. The polymerase thus purified contained L (large protein) and NS proteins as the intrinsic subunits and multiple species of enzyme were found which differ in the molar ratio of L to NS. Since the enzyme with the highest activity was composed of equimolar amounts of the two subunits and exhibited the sedimentation coefficient of approximately 11 S in a buffer containing 0.2 M NaCl, the structure of active protomer was suggested to be (L) \( \cdot \) (NS). In accordance with this conclusion, enzyme preparations deficient in the content of NS protein, were activated by the addition of NS protein.

The purified RNA polymerase catalyzed the synthesis of poly(A), which was covalently attached to the 3' termini of RNA products, and RNA, only in the presence of all 4 substrates. The present finding might be the first which indicates that the transcriptase itself catalyzes post-transcriptional modification of mRNA by adding poly(A) sequences to the 3'-OH termini. The molecular mechanism of the switch from transcription to poly(A) synthesis, however, remains to be investigated.

Vesicular stomatitis virus, the prototype of the rhabdovirus group, contains a single strand RNA of 3.6 to 4.0 \( \times 10^6 \) daltons as the viral genome which is complementary to messenger RNA synthesized in virus-infected cells (1, 2). Upon infection to susceptible cells, VS virus exhibits two types of RNA metabolism: mRNA transcription, and replication of the viral genome. It has been established that the synthesis of mRNA is catalyzed by a virion-associated RNA-dependent RNA polymerase (ribonucleoside triphosphate: RNA nucleotidyltransferase (EC 2.7.7.6)), at least during the initial stage of virus multiplication. The products transcribed are monocistronic, and two species of mRNA with the sedimentation coefficients of 12 to 18 S and 28 to 31 S have been identified as the major components (3-5). Like most eukaryotic mRNA, they possess polyadenylic acid (poly(A)) sequences covalently linked to the 3'-OH termini (6-8). Since RNA synthesized in vitro in purified virions is attached with poly(A) sequences (9, 10), and since viral RNA contains no poly(U) stretches to code poly(A) (11-13), an enzyme activity is expected to exist in virions which catalyzes polymerization of adenylate in the absence of template.

On the other hand, replication of the genome RNA seems to require the continuous supply of unstable protein(s) synthesized in infected cells (14, 15) and the product is 40 S negative-strand RNA which is not attached with poly(A) sequence (16).

In order to reveal the molecular mechanism underlying the multiplication of VS virus-RNA as outlined above, we tried to purify the enzyme system which catalyzes transcription and poly(A) synthesis from the New Jersey serotype of VS virus, and to identify the molecular structure as well as the enzymatic properties. It has been well established that VS virions contain at least five separate structural polypeptides: L (large protein; \( M_r = 190,000 \)); G (glycoprotein; \( M_r = 69,000 \)); N (nucleoprotein; \( M_r = 50,000 \)); NS (\( M_r = 40,000 \) to 45,000); and M (membrane protein; \( M_r = 29,000 \)) (17). Since a viral subparticle which is composed of the RNA, N, L, and NS proteins is capable of synthesizing RNA in vitro was isolated (18), these three proteins have been believed to be sufficient for transcription, whereas the two envelope proteins, G and M, are not essential. The dissociation and reconstitution studies by Wagner and his colleagues (19) revealed that the L protein and the N protein-RNA complex were required for transcription. In this report, we propose that both of the polypeptides L and NS are required for N-RNA complex-directed RNA synthesis and, moreover, the RNA polymerase itself catalyzes post-transcriptional addition of poly(A) to the product RNA. During the preparation of the manuscript, Emerson and Yu (20) and Imblum and Wagner (21) reported...
evidence indicating that not only L but also NS protein are
required for transcription with the Indiana serotype of VS
virus.

A preliminary report of this study has been published previously (22).

MATERIALS AND METHODS

Chemicals—Unlabeled ribonucleoside triphosphates were purchased from Sigma Chemical Co., P-L Biochemical Co., and Boehringer GmbH, while [3H]-labeled nucleosides were from Schwarz/Mann. [γ-32P]ATP was generously provided by Dr. M. Imai. [3H]NaBH₄ was a product of New England Nuclear Co. Cesium chloride, cesium sulfate, and recrystallized products of sodium dodecyl sulfate (SDS) and N, N'-methylene-bisacrylamide were obtained from Nakarai Chemicals. Dithiothreitol, Triton X-100, recrystallized acrylamide, and urea were from Wako Chemical Co. Nonidet P-40 (NP40) was a product of Shell Chemical Co. DEAE-Sephadex A60 and phosphocellulose P11 were products of Pharmacia and Whatman, respectively. Pancreatic ribonuclease (EC 3.2.1.2) and bovine liver catalase (EC 1.11.1.6) were obtained from Sigma Chemical Co., ribonuclease T₁ (EC 3.1.4.12) and T₂ (EC 3.1.4.22) were obtained from Sigma Co. and Boehringer GmbH, respectively.

Virus and Cell—Stock preparations of VS virus (New Jersey serotype) and BHK cell were supplied by Dr. A. Kawai of this institute. BHK cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% tryptose phosphate broth and 5% calf serum. Monolayer cultures were infected with VS virus at the multiplicity of 10³ pfu per cell and virus was grown at 37°C for 15 to 20 hours. Culture fluid obtained was clarified from cellular debris by centrifugation at a Sorvall GS-3 rotor at 4°C and 7,000 rpm for 30 min. To one liter of supernatant fluid, 25 g of NaCl and 75 g of polyethylene glycol 6000 were added according to the procedure of Roy and Bishop (23), and precipitated formed after standing overnight at 4°C were collected by centrifugation at 7,000 rpm for 30 min. Pellets were then suspended in 0.01 M Tris-HCl (pH 7.8 at 4°C), 0.1 M NaCl, and centrifuged at 30,000 rpm for 5 hours at 4°C. After two cycles of centrifugation, virus bands obtained were collected and dialyzed against 0.1 M Tris-HCl (pH 7.8 at 4°C), 0.1 mM dithiothreitol and 10% glycerol, and stored at 4°C until use.

Enzyme Assays—Standard reaction mixture for RNA polymerase assay contained in 0.25 ml: Tris-HCl buffer (pH 8.3 at 25°C), 30 mM magnesium acetate, 1.25 mM dithiothreitol, 1.25 mM NaCl, 17.5 μM EDTA, 1 μM each of GTP, CTP, UTP, and [8-3H]ATP (specific activity, 1.1 × 10⁸ cpm per nmol); template; and enzyme. When intact virions were used as the enzyme source, 20 μg of nonionic detergent (NP40 or Triton X-100) was added to this reaction mixture. In some experiments, [8-3H]GTP or [5-3H]UTP (specific activity, 1.1 × 10⁸ cpm per nmol) was used as the labeled substrate in place of [8-3H]ATP.

Catalase activity was assayed by measuring the disappearance of the absorbance at 240 nm of H₂O₂, whereas β-galactosidase activity was monitored optically by hydrolysis of α-nitrophenyl-β-galactoside.

Determination of Polycl.—After incubation for RNA synthesis, the reaction mixtures were chilled and diluted 4-fold with 0.1 M sodium phosphate buffer (pH 7.0); NaCl and EDTA were added to the final reaction concentrations of 0.3 mM and 42 μM, respectively. After RNA polymerase was inactivated by heating at 100°C for 1 min, product RNA was digested with pancreatic ribonuclease (25 μg per ml) and ribonuclease T₁ (25 units per ml) for 30 min at 37°C, and undigested acid insoluble radioactivity was determined.

Purification of RNA Polymerase—Virions were treated with 1% Triton X-100 in low ionic strength buffer which consists of 10 mM Tris-HCl (pH 7.8 at 4°C), 0.1 mM NaCl, 5 mM EDTA, and 2.5 mM dithiothreitol at 30°C and 0°C each for 10 min. Three milliliters each of the disrupted virions was layered on 2 ml of discontinuous Cs₂SO₄ solution (1.5 ml of 0.25 M Cs₂SO₄ solution on 0.5 M CsCl cushion) and centrifuged in a Spinco SW50.1 rotor at 1°C for 4 hours at 60,000 rpm. Virus cores concentrated on 1 M Cs₂SO₄ cushion were collected and dialyzed against Buffer V (10 mM Tris-HCl (pH 7.6 at 4°C), 0.1 mM NaCl, 1 mM dithiothreitol, and 10% glycerol). Dialyzed cores were further treated with 1.5 M CsCl and 10 mM dithiothreitol at 30°C and 0°C each for 10 min, and 2-ml portions were centrifuged on 5.5 ml of discontinuous CsCl gradients (2.5 ml of 1.75 M CsCl on 1-ml 5 M CsCl cushion) in a Spinco SW50.1 rotor at 3°C for 4 hours at 40,000 rpm. Solutions of Cs₂SO₄ and CsCl were prepared by diluting 1 M Cs₂SO₄ or 5 M CsCl solutions with 20 mM Tris-HCl (pH 7.6 at 4°C), 1 mM dithiothreitol, and 20% glycerol. After centrifugation, each fraction was tested for RNA polymerase and template activities. Enzyme fractions at 4°C were combined and dialyzed against Buffer V containing 30% glycerol, whereas ribonuclease protein template fractions were combined and dialyzed against Buffer V for use. In some experiments, the template fractions were further purified to remove endogenous RNA polymerase by recentrifugation in CsCl gradients as above, or otherwise endogenous enzyme activity was inactivated by treatment with freshly deionized 6 mM urea at 30°C for 10 min and urea was removed by dialysis against Buffer V at 4°C.

RNA polymerase fractions thus obtained were subjected to further purification by regular purification procedures, such as glycerol gradient centrifugation or chromatography on DEAE-Sephadex or phosphocellulose column.

Terminal Labeling of RNA—5'-Terminal nucleotides of RNA were labeled by performing RNA synthesis in vitro in the presence of [γ-32P]ATP as a labeled substrate whereas 3'-terminal nucleotides were labeled using the NaIO₄ oxidation/[3H]NaBH₄ reduction technique (24, 25). To label the 3'-OH of terminal nucleotides, the reaction mixture for RNA synthesis was treated with 0.1% SDS, dialyzed against 0.05 M sodium acetate buffer (pH 5.5) containing 0.1% SDS, and treated with 0.05 M NaIO₄ in the dark. After removal of excess NaIO₄ by dialysis against sodium acetate-SDS buffer, 0.2 μCi of [3H]NaBH₄ (specific activity, 162 mCi/mmol) dissolved in 0.1 M NaOH was added, and the pH was immediately adjusted to 8 by adding 1 M KH₂PO₄. The reduction was allowed to proceed for 3 hours at room temperature.

RESULTS

Requirements for RNA Polymerase Reaction

In the course of the systematic search for the optimum conditions of RNA synthesis by RNA polymerase in VS virus, a few new features were found: the maximum activity was observed after treatment of virus preparations with 0.1% Nonidet P40 (NP40); the optimum pH for the reaction was 8.3, which was higher than the value (pH 7.3) reported by Huang et al. (26); although Mn²⁺ ion at concentrations over 2 mM was unable to substitute for Mg²⁺ ion, but rather inhibited the Mg²⁺-dependent reaction as reported by others (26, 27), the substitution with Mn³⁺ at concentrations below 2 mM permitted a considerable level of RNA synthesis. The maximum activity obtained with 1 mM Mn³⁺, however, was only one-third of that with 5 mM Mg²⁺ ion. Such Mn³⁺-dependent reaction has been demonstrated independently for the in vitro synthesis of RNA by disrupted virions of Newcastle disease virus (28), and ribonucleocapsids isolated from rhabdovirus-infected cells (29). The reaction was very sensitive to salt (NaCl) concentration with a sharp optimum at 0.07 M, and such salt sensitivity was observed even with the purified RNA polymerase (DEAE-Sephadex fraction). Thus, the concentrations employed in other reports, 0.1 M (30) or 0.15 M NaCl (18), lead to considerable suppression of enzyme activity. Since the polymerase activity was rather reduced at temperatures higher than 30°C, all the reactions described were performed at 30°C.

Solubilization of RNA Polymerase from Virions

Treatment of VS virions with nonionic detergent, Triton X-100, in the presence of dithiothreitol at low ionic strength leads to solubilization of viral envelopes. When disrupted virions were centrifuged on a discontinuous gradient of cesium sulfate, solubilized envelope lipids and proteins remained on the top of gradient while the viral core particles sedimented on the cushion of 1 M Cs₂SO₄. Among the Cs₂SO₄ fractions thus obtained, RNA polymerase activity was found only in the core

1 We thank Dr. A. Kawai for his advice and assistance in preparation of VS virus in quantities.
fractions indicating that RNA polymerase protein(s) remained bound in core particles. When the polypeptide composition of the core particles as well as of the original virus preparation was analyzed by polyacrylamide gel electrophoresis in the presence of SDS, the proteins L, N, NS, and a portion of M were found in the core particles (Fig. 1).

The cores were further dissociated by exposure to a high ionic strength buffer containing 1.5 M CsCl and subjected to centrifugation in a discontinuous gradient of CsCl from 1.75 to 3 M; the dissociated proteins were separated from ribonucleoproteins and recovered in the top fraction. Neither the top fraction nor the ribonucleoprotein fraction alone exhibited RNA polymerase activity when tested separately. However, upon combining the two fractions, the activity was restored quantitatively (Fig. 2). When the ribonucleoprotein template containing 23 µg of N protein was assayed by adding the top fraction, the activity increased linearly at least up to 10.3 µg of the dissociated protein. Polyacrylamide gel electrophoresis revealed that the dissociated protein fraction contained the L, NS, M proteins, and a small amount of N protein, while the majority of N protein remained attached in the ribonucleoprotein (RNP) fraction as a sole component (Fig. 1). A trace amount of L protein often accompanied the RNP fraction which seemed to cause a low but significant level of endogenous RNA polymerase activity.

In the enzyme reconstitution experiment as noted above, no activity was found when viral RNA purified by SDS-phenol extraction method was used as a template in place of the RNP fraction (Table I) suggesting that the N protein is absolutely required for RNA to be transcribed by the enzyme. Neither synthetic polynucleotides including poly(A), poly(U) (Table I), poly(C), and poly(G) (data not shown), nor natural nucleic acids as Escherichia coli ribosomal RNA and T7 DNA exhibited template activity (Table I). Moreover, attempts to replace the N protein by polyamines or histones have also been unsuccessful.

**Purification of RNA Polymerase**

**Glycerol Gradient Centrifugation**—In order to identify the polypeptide component(s) necessary for RNA polymerase activity, the proteins dissociated by CsCl were further fractionated by centrifugation through a 15 to 35% glycerol gradient in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 M NaCl, 1 mM dithiothreitol and 0.2% Triton X-100. As shown in Fig. 3, a peak of enzyme activity was found only when assayed by adding the RNP template. The peak fraction of enzyme activ-

**Fig. 1 (left).** Polypeptide composition of each fraction of enzyme purification. (A) VS virion (11.4 µg of protein); (B) core particle (5.9 µg of protein); (C) RNA polymerase (4.7 µg) purified by glycerol gradient centrifugation; and (D) ribonucleoprotein (5.4 µg of protein) purified by two cycles of CsCl centrifugation. Samples were treated with 0.5% SDS and 25 mM dithiothreitol for 10 min at 37°C and analyzed by polyacrylamide gel (7.5%) electrophoresis in the presence of SDS. Gels were stained with Coomassie brilliant blue and after destaining, scanned with Joyce-Loebl microdensitometer MKIII. Electrophoresis was from right to left.

**Fig. 2 (center).** Cesium chloride centrifugation of dissociated core. Core particles (3.1 mg of protein) were treated with 1.5 M CsCl and centrifuged in a discontinuous gradient of CsCl as described under "Materials and Methods." After centrifugation, RNA polymerase activity was determined with 5 µl each of the fraction (O---O), 5 µl each of the fractions supplemented with 5 µl of the fraction number 3 (---), or 5 µl each of the fractions supplemented with 5 µl of the fraction number 10 (-----). RNA synthesis was performed for 1 hour at 30°C. Centrifugation was from right to left.

**Fig. 3 (right).** Glycerol gradient centrifugation of solubilized RNA polymerase. RNA polymerase was solubilized from core particles as described in Fig. 2, and dialyzed to remove CsCl against Buffer V. Two milliliters of the dialyzed enzyme containing 1.76 mg of protein was centrifuged on 88 ml of 15 to 35% glycerol gradient in 0.1 M sodium phosphate buffer (pH 7.0) containing 1 M NaCl, 0.2% Triton X-100, and 1 mM dithiothreitol in a Spinco SW25.2 rotor for 78 hours at 24,000 rpm at 4°C. After centrifugation, RNA polymerase activity (-----) of 25 µl each of the glycerol fractions was determined using ribonucleoprotein containing 23 µg of N protein as the template. Protein distribution was analyzed by SDS-polyacrylamide gel electrophoresis of 50 µl each of the glycerol fractions (L protein, ----; M protein, -----; NS protein, ---; N protein, ----).
Where indicated, 10 μg of histone, spermidine, or poly(L-ornithine) 0.13 A260 unit; Escherichia coli ribosomal RNA, 0.3 A260 unit; poly(A), RNA purified by SDS-phenol methods, 0.073 A260 units; T7 DNA, acids were added: VS virus nucleoprotein, 13 μg of protein; VS virus-polymerase solubilized from VS virus core particles. For each assay, 94 was added. RNA synthesis was carried out at 30°C for 4.5 hours.

Heated RNA preparations were prepared by incubation for 5 min at 100°C followed by rapid cooling. CsCl top fraction formed aggregates with decreased ionic strength and was recovered in the flow-through fraction. The DEAE-Sephadex column. The M protein contaminating the preparations.

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| Template activity of various nucleic acids |
|-------------------------------------------|
| Template                                   | [PH]AMP incorporated | cpm  |
| VS virus ribonucleoprotein (RNP)            | 19,016                |
| VS virus RNA                               | 132                   |
| VS virus heated RNA                        | 122                   |
| VS virus RNA + spermidine                  | 6                     |
| VS virus RNA + spermidine + poly(L-ornithine) | 196                 |
| T7 phage DNA                               | 360                   |
| E. coli ribosomal RNA                      | 209                   |
| Poly(A)                                    | 140                   |
| Poly(U)                                    | 232                   |
| Poly(A) + poly(U)                          | 1,191                 |
| None                                       | 0                     |

TABLE 1

Template activity of various nucleic acids was tested with RNA polymerase solubilized from VS virus core particles. For each assay, 94 μg of solubilized RNA polymerase and following amounts of nucleic acids were added: VS virus nucleoprotein, 13 μg of protein; VS virus-RNA purified by SDS-phenol methods, 0.073 A260 unit; Escherichia coli ribosomal RNA, 0.3 A260 unit; poly(A), RNA purified by SDS-phenol methods, 0.073 A260 units; T7 DNA, acids were added: VS virus nucleoprotein, 13 μg of protein; VS virus-polymerase solubilized from VS virus core particles. For each assay, 94 was added. RNA synthesis was carried out at 30°C for 4.5 hours.

The line represented in Fig. 5 clearly indicates that the enzyme activity is maximal when the two proteins are associated to form a complex at the molar ratio of 1:1. Since

Fig. 4. DEAE-Sephadex column chromatography of solubilized RNA polymerase. RNA polymerase was solubilized from core particles as described in Fig. 4, and dialyzed against 0.05 M sodium phosphate buffer (pH 7.6) containing 0.1 M NaCl, 0.2% Triton X-100, 1 mM dithiothreitol, and 30% glycerol. Dialyzed enzyme was applied onto a DEAE-Sephadex A50 column (0.9 x 6.5 cm) and eluted first with the buffer containing 0.1 M NaCl followed with the buffer containing 0.5 M NaCl.

Among the three protein peaks, RNA polymerase activity was found only in peaks I and II when assayed with urea-treated RNP as the template. As an approach to understanding whether both of the L and NS proteins are absolutely required for enzyme activity, the activity of enzyme fractions expressed per 1 μg of L protein was plotted against the ratio of NS to L, and the result is shown in Fig. 5. In this experiment, the enzyme activities were determined on the basis of the maximum activities obtained by adding saturated amounts of the template. The line represented in Fig. 5 clearly indicates that the enzyme activity is maximal when the two proteins are associated to form a complex at the molar ratio of 1:1. Since

the enzyme purification are eluted in peaks I and III. In fact, chromatography of each peak fraction on second DEAE-Sephadex columns yielded a main peak, which was eluted at the salt concentration similar to that required for the first chromatography, but in addition shoulders and small peaks of cross-contaminants or dissociated products (Fig. 4, B to D). Since the NS content in the enzyme fractions decreased upon rechromatography, it should be considered that L-NS complexes were gradually dissociated into the individual components during such manipulations. Better separation of the three peaks was always achieved when elution was performed with phosphate buffer rather than with Tris buffer.

Among the three protein peaks, RNA polymerase activity was found only in peaks I and II when assayed with urea-treated RNP as the template. As an approach to understanding whether both of the L and NS proteins are absolutely required for enzyme activity, the activity of enzyme fractions expressed per 1 μg of L protein was plotted against the ratio of NS to L, and the result is shown in Fig. 5. In this experiment, the enzyme activities were determined on the basis of the maximum activities obtained by adding saturated amounts of the template. The line represented in Fig. 5 clearly indicates that the enzyme activity is maximal when the two proteins are associated to form a complex at the molar ratio of 1:1. Since

DEAE-Sephadex Column Chromatography—Attempts were also made to fractionate the dissociated proteins by chromatography on a DEAE-Sephadex column. The CsCl top fraction previously dialyzed against a low salt buffer consisting of 0.05 M sodium phosphate (pH 7.6), 0.1 M NaCl, 1 mM dithiothreitol, 0.2% Triton X-100, and 30% glycerol was applied onto a DEAE-Sephadex column. The M protein contaminating the CsCl top fraction formed aggregates with decreased ionic strength and was recovered in the flow-through fraction. The absorbed materials containing RNA polymerase activity were eluted with a high salt buffer containing 0.5 M NaCl, and subjected to the second DEAE-Sephadex chromatography. Elution was performed with a linear gradient of NaCl from 0.1 to 0.5 M. As shown in Fig. 4A, there appeared three separate peaks. The first peak (peak I) contained the L and NS proteins at an approximate molar ratio of 1:0.2 to 0.5. The second peak (peak II) contained the two proteins at a molar ratio of 1:0.9 to 1.0, and the third peak (peak III) contained mainly the NS protein. Since VS virions contain approximately equimolar amounts of the two polypeptides, both of which are quantitatively released from the core particles by CsCl, it appears reasonable to assume that peak II represents the native enzyme structure of L-NS complex, and products dissociated during

* S. Naito, unpublished observation.
the line intercepts zero point, the L protein alone seems inactive in catalyzing RNA synthesis.

When the purified enzyme with maximum activity (peak II) was centrifuged through glycerol gradients in either low or high ionic strength buffer, it sedimented to the position corresponding to the sedimentation coefficient of 10.8 S in 0.2 M NaCl and 13.5 S in 1.0 M NaCl, respectively (Fig. 6). On the basis of the sedimentation coefficients and the molecular weight of L and NS proteins, we tentatively propose that the native form of VS virus RNA polymerase in low ionic strength buffer is (L)\textsubscript{1} (NS)\textsubscript{1}, which forms a dimeric structure in the presence of 1.0 M NaCl.

Phosphocellulose Column Chromatography—As an effort to confirm the above interpretation, the effect of NS protein on varieties of RNA polymerase preparation was tested. NS protein was isolated to homogeneity by phosphocellulose column chromatography of proteins released from cores by CsCl. The NS protein was recovered in the flow-through fraction, while varieties of L:NS complex were eluted at increased salt concentrations forming broad peaks (data not shown). RNA polymerase activity was again observed only in fractions containing both L and NS proteins. The effect of NS protein on RNA polymerase preparations containing the L and NS proteins at different ratios was then analyzed, and parts of the result are shown in Table II. Significant stimulation by NS protein (4.9 ng/ml) of RNA synthesis catalyzed by 0.03 ml of peak I enzyme (15.3 pg of L and 1.7 pg of NS protein per ml; NS/L molar ratio = 0.47), or 0.02 ml of peak II enzyme (23.5 pg of L and 5.2 pg of NS protein per ml; NS/L molar ratio = 0.93) was examined in the standard reaction mixture containing urea-treated RNP template (18.4 pg of protein). Incubation was carried out at 30° for 4 hours.

| RNA polymerase | NS protein | [\textsuperscript{3}H]AMP incorporated | Stimulation | NS:L molar ratio |
|----------------|------------|------------------------------------|-------------|----------------|
| Peak I         | 0          | 5,156 cpm                          | 1.00        | 0.47           |
|                 | 0.05       | 7,447 cpm                          | 1.44        | 2.52           |
|                 | 0.10       | 8,964 cpm                          | 1.74        | 4.61           |
| Peak II        | 0          | 8,186 cpm                          | 1.18        | 3.14           |
|                 | 0.10       | 7,783 cpm                          | 1.23        | 5.38           |

Poly(A) Synthesis by RNA Polymerase

Although purified virions are able to synthesize RNA containing poly(A) which is covalently attached to the 3'-OH terminus of product RNA (8), no poly(A) is synthesized in the presence of ATP as a sole substrate (9, 10). With use of disrupted virions, 30 to 35% of radioactivity of the product RNA formed remained acid-insoluble after treatment with ribonuclease when [\textsuperscript{3}H]ATP was used as the labeled substrate. When [\textsuperscript{3}H]UTP or [\textsuperscript{3}H]GTP was employed as the labeled substrate, almost all the radioactivity was rendered into acid-soluble forms by ribonuclease treatment (22). It has been believed that such poly(A) sequences covalently attached to RNA are synthesized by an enzyme system different from the
transcriptase, and a poly(A) polymerase was indeed isolated from vaccinia virus (31, 32). In contrast, the purified VS virus RNA polymerase itself exhibited poly(A) synthesizing activity. The synthesis of poly(A) sequences by the purified RNA polymerase also required the presence of all four triphosphates. It is worthwhile to note that the ribonuclease-resistant fraction of product RNA synthesized in the presence of \( \text{Mn}^{2+} \) was 10 to 15%, which is considerably lower than that of RNA synthesized in the regular reaction mixture containing \( \text{Mg}^{2+} \) as a divalent metal cofactor (Fig. 7). The kinetic profile of the appearance of ribonuclease-resistant poly(A) sequence catalyzed by disrupted virions (data not shown).

Product RNA synthesized by the purified RNA polymerase exhibited a sedimentation coefficient of approximately 16 S as a main component and the ribonuclease-resistant poly(A) was also found in this peak (Fig. 8A) indicating that the poly(A) is associated to this high molecular weight RNA. In fact, the poly(A) gave a peak of 4 to 5 S when RNA was centrifuged after treatment with ribonuclease (Fig. 8B). The molecular size and poly(A) content of product RNA synthesized on the urea-column chromatography as described in the text. RNA synthesis was performed with 0.05 ml of RNA polymerase (11.4 pg of L and 2.2 pg of NS protein per ml) in the presence or absence of 0.42 Kg of NS protein. After incubation at 30° for 2 hours, the reaction mixtures were treated with ribonuclease (Fig. 9A). The reaction mixtures were divided into two equal portions, one of which was treated with ribonuclease for the determination of poly(A) content.

**TABLE III**

| Labeled substrate | NS protein | \(^{3}H\)AMP incorporated | Rnase-resistant |
|-------------------|------------|--------------------------|----------------|
| ATP               | –          | 3,418, 913               | 26.7           |
| ATP               | +          | 5,726, 1,472             | 24             |
| GTP               | –          | 1,752                    | 7              |
| GTP               | +          | 2,425                    | 1.0            |

Fig. 7. Time course of the synthesis of RNA and poly(A) by purified VS virions. RNA polymerase reaction catalyzed by purified VS virions (23.1 µg of protein) was performed according to the standard procedure as described under “Materials and Methods” using \(^{3}H\)ATP as the labeled substrate, and in the presence of 5 mM \( \text{Mg}^{2+} \) or 1 mM \( \text{Mn}^{2+} \) as the divalent metal cofactor. The amounts of RNA and poly(A) synthesized were determined at the times indicated: \( \text{Mg}^{2+} \)-dependent reaction (O—O, RNA; •—•, poly(A)); \( \text{Mn}^{2+} \)-dependent reaction (□—□, RNA; ■—■, poly(A)).

**Discussion**

Of the several types of virion-associated nucleic acid polymerases, extensive purification and analysis of enzymatic properties as well as molecular structure have been performed only on the reverse transcriptase (the RNA-dependent DNA polymerase) from oncomaviruses (33, 34). In contrast, however, little is known about the DNA- and RNA-dependent RNA polymerases present in wide varieties of viruses including poxviruses, diplomaviruses, myxoviruses, paramyxoviruses, and rhabdoviruses mainly due to the difficulty of obtaining highly purified enzymes in quantities.

Isolation of nucleocapsid cores containing RNA polymerase activity from VS virus was first achieved by either polyethylene glycol-dextran phase separation or agarose gel chromatography of virions disrupted with a nonionic detergent Triton N-101 (35). Similar ribonucleoprotein, which retained infectivity as well as RNA polymerase activity, was obtained by disruption of virions with Triton N-101 in the presence of 0.5 M CsCl followed by isolation of the ribonucleoprotein by centrifugation in sucrose gradient (36). According to these procedures the two envelope proteins, G and M, were completely solubilized and could be removed from the ribonucleoparticle. On the other hand, Emerson and Wagner (18) reported that the transcriptase activity was dissociated from virions of the Indiana serotype VS virus by treatment with high ionic strength buffer containing Triton X-100 and was separated from the template ribonucleoparticle by centrifugation through sucrose gradients. Solubilized proteins they obtained, however, contained not only the polymerase but also all the envelope proteins, and it seemed difficult to isolate pure enzyme by subsequent purification employing conventional techniques of enzyme purification (19).

As reported previously (37–39), equilibrium centrifugation in CsCl or Cs_2SO_4 is useful for dissociating RNA polymerase from DNA so long as they are not in the process of RNA synthesis. Moreover, it has been established that CsCl is more potent than Cs_2SO_4 in this dissociation since a part of the polymerase, which was bound to DNA regions with weak affinity but were still recovered in the cesium-sulfate complex, was further dissociated from the DNA by exposure to CsCl.
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Purified RNA polymerase preparations with the highest activity contained equimolar amounts of the L and NS proteins; the activity of enzyme preparations containing less NS protein (NS/L < 1) was always lower and was considerably stimulated by the addition of NS protein, which alone was inactive in RNA synthesis. Thus, the enzyme activity expressed per unit amount of L protein is proportional to NS content up to 1:1 in molar ratio (Fig. 5). On the basis of these observations, we tentatively propose that both the L and NS proteins are the intrinsic subunits of VS virus-RNA polymerase and the native enzyme consists of equimolar amounts of the two polypeptides. Although Emerson and Wagner (19) succeeded in preparing NS-free L protein as well as pure NS protein from the Indiana serotype of VS virus, our attempts have been unsuccessful to isolate L protein from the New Jersey serotype employed in this research, supposedly due to differences in the conformation of L-NS complexes between the two strains of VS virus. The difference in the structure of the genetic elements has also been proposed between the two strains because six complementation groups were identified for the New Jersey serotype of VS virus (41), whereas the Indiana serotype contains only five complementation groups (42).

Sedimentation velocity analysis indicated that the functional form in low ionic strength buffers is apparently (L)₂(NS)₂, associating to form dimer (L)L(NS)₂, in the presence of high concentrations of salt. In concert with this interpretation, approximately equal amounts of the L and NS proteins were found in purified virions. Although a number of enzyme preparations are known to form polymers in the absence of salt such as the DNA-dependent RNA polymerase from E. coli (43), it is also known that enzymes such as the Q₂-RNA replicase, the RNA-dependent RNA polymerase from Q₂ phage-infected E. coli cells, dissociate at decreased ionic strength (44).

During the purification of VS virus-RNA polymerase, it was recognized that the poly(A) synthesizing activity could never be separated from the transcriptase activity and that the purified RNA polymerase itself directed the synthesis of RNA containing poly(A) sequence. The content of poly(A) in product RNA synthesized by enzymes of different purification steps was always constant indicating that the transcriptase itself catalyzes the post-transcriptional addition of poly(A) sequence. If this be the case, the transcriptase must recognize a unique structure of the 3′-OH terminus of product RNA or of the signal on the termini of transcription unit in the template RNA. It should also be considered that poly(A) could be synthesized by repeated reading of oligo(U) stretches if such sequences exist at the termini of transcription unit on the template RNA (8). Such a template-slippage mechanism of poly(A) synthesis was proposed for the DNA-dependent poly(A) synthesis by the bacterial RNA polymerase (45).

Acknowledgments—The authors are grateful to Dr. Ryuji Fukuda who initiated this research and provided advice during the course of this work.

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Fig. 8 (left). Sucrose gradient centrifugation of product RNA synthesized by purified VS virus-RNA polymerase. RNA synthesis was carried out in a 0.5 ml reaction mixture containing 7.2 µg of purified RNA polymerase (glycerol gradient fraction) and ribonucleoprotein template (45.6 µg of N protein). After incubation for 1 hour at 30°, the reaction mixture was divided into two equal portions; one part was treated with 0.4% SDS at 37° for 5 min (A), while the other portion was treated with ribonuclease prior to SDS treatment (B). Samples were centrifuged on 12 ml of 15 to 35% sucrose in 0.01 M Tris-HCl (pH 7.4) at 4°/0.15 M NaCl buffer in a Spinco SW41 rotor at 40,000 rpm for 18 hours. The sucrose fractions of Experiment A were divided into equal halves, and acid-insoluble radioactivity was determined before (O—O) or after (O—O) ribonuclease treatment, while in Experiment B acid-insoluble radioactivity was determined with whole fractions. As the reference for determination of sedimentation coefficient of the products, 14C-labeled Escherichia coli total RNA centrifuged in a separate tube. Centrifugation was from right to left.

Fig. 9 (right). Sucrose gradient centrifugation of terminally labeled RNA. RNA synthesis was carried out in 0.5 ml using 0.84 µg of purified RNA polymerase (glycerol gradient fraction) and ribonucleoprotein template (15.8 µg of N protein). The reaction mixture contained 0.21 mM [γ-32P]ATP (specific activity, 7 × 10⁶ cpm/nmol) in place of [γ-3H]ATP. After incubation for 3 hours at 30°, 5 µl of 10% SDS was added and the reaction mixture was dialyzed first against 0.15 M sodium acetate buffer (pH 5.5) containing 0.1% SDS, and then against sodium acetate buffer containing 0.5% NaIO₄ in the dark. After removal of excess NaIO₄ by dialysis against sodium acetate buffer, RNA was treated with 2 mM [3H]NaBH₄ (specific activity, 7 × 10⁶ cpm/nmol) for 3 hours in the dark. Labeled RNA was centrifuged on 11 ml of 15 to 35% sucrose in 0.01 M Tris-HCl (pH 7.4) at 4°/0.15 M NaCl buffer in a Spinco SW41 rotor at 35,000 rpm for 16 hours. The sucrose gradient was fractionated into 32 tubes and acid-insoluble radioactivities in 0.15 ml portions were determined before (A) or after (B) ribonuclease treatment. As the reference for determination of sedimentation coefficient of the products, 3H-labeled Escherichia coli total RNA centrifuged in a separate tube. Centrifugation was from right to left. 3H radioactivity, O—O; 32P radioactivity, O—O.

These two lines of investigation led us to develop the enzyme purification procedure described in this report, such that the proteins in viral particles are dissociated stepwise on the basis of their affinity to the genome RNA. Recently a procedure for the isolation of reverse transcriptase from avian myeloblastosis virus (40). In contrast to the finding by Emerson and Wagner (19), the enzyme solubilized by the present procedure was not unstable, providing that the appropriate concentrations of Triton X-100 (0.2%), dithiothreitol (1 mM), and glycerol (30%) were included in all buffers used during the enzyme purification. Thus, further purification could be carried out by several conventional techniques of enzyme purification such as...
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