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Properties of a Soluble DNA Polymerase Isolated from Rous Sarcoma Virus

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ABSTRACT **The DNA polymerase of the Prague strain of Rous sarcoma virus of subgroup C and of the Schmidt-Ruppin strain of subgroup A has been solubilized. DNA polymerase purified by sucrose gradient sedimentation and chromatography on DEAE-cellulose represented less than 2% of the soluble [^{14}C]protein of the virus. The enzyme was separated from 90% of the viral glycoprotein; it is probably different from the viral group-specific antigen. The sedimentation coefficient ($s_{20,w}$) of the soluble DNA polymerase was 8 S before, and 6 S after, incubation with pancreatic RNase. The molecular weight of the 8S DNA polymerase was estimated to be about 170,000, and that of the 6S DNA polymerase to be about 110,000.**

Purified DNA polymerase had a high activity with 60-70S viral RNA or salmon DNA as template, but it had a low activity with heat-dissociated 60-70S RNA, influenza virus RNA, or the RNA of tobacco mosaic virus as template. Neither the 8S nor the 6S DNA polymerase had endogenous template activity. The DNA-dependent and the RNA-dependent DNA polymerase activities of the Prague strain coincided in sucrose gradients, both in the 8S and the 6S form. It is concluded that the RNA-dependent and the DNA-dependent DNA polymerase activities of the avian tumor viruses are probably due to the same enzyme.

The DNA polymerase associated with avian and murine RNA tumor viruses (1, 2) accepts different kinds of nucleic acids as templates. The enzyme was originally found to be RNA-dependent with endogenous viral RNA as template (1-3). Later, it was found that both native and denatured double-stranded DNAs from various sources (4-8), as well as synthetic nucleic acids (4, 9), could serve as templates. It was therefore of interest whether the enzyme exhibits template specificity for natural RNAs, i.e., whether it prefers tumor virus RNA over RNAs from other sources; whether the DNA polymerase activities primed by endogenous viral RNA, by natural DNAs, or by synthetic nucleic acids are common to a single enzyme, to modifications of one enzyme, or to different enzymes was also of interest. It had also not been determined whether the viral DNA polymerase corresponds to any of the known structural proteins of the virus. Such questions may be answered by means of a soluble and purified viral DNA polymerase. This report describes the isolation and some properties of a soluble DNA polymerase from two strains of Rous Sarcoma Virus (RSV).

MATERIALS AND METHODS

Solutions

Standard buffer contains 0.1 M NaCl-0.01 M Tris (pH 7.4)-1 mM EDTA; low-salt buffer for DEAE-cellulose chromatography is 0.05 M Tris (pH 7.4)-0.01 M $MgCl_2$-0.02 M KCl-0.2 mM EDTA-1 mM dithiothreitol (DTT)-5% glycerol; disruption buffer is 0.01 M Tris (pH 8.0)-2 mM EDTA-1 mM DTT-0.02 M KCl (for DEAE-cellulose chromatography) or 0.1 M KCl (for sedimentation).

Viruses

Growth, radioactive labeling, and purification of PR RSV-C and SR RSV-A followed published procedures (10-12). Medium for amino acid labeling (10) was modified to contain 1% calf serum, 1% chick serum, 1% dimethyl sulfoxide, and the glutamine concentration of normal medium 199. After appropriate dilution with standard buffer, purified virus was layered over a 2-cm column of 30% glycerol containing 0.01 M Tris (pH 8.0)-1 mM EDTA-1.5 mM 2-mercaptoethanol and centrifuged in a Spinco SW50.1 rotor for 30 min at 46,000 rpm (at 5°C) onto a cushion of 85% glycerol containing the same buffer. The virus, collected at the interface, was stored at −20°C. Virus concentration was determined by measuring the A_{260} in standard buffer containing 0.2% sodium dodecyl sulfate; the (SDS) A_{260}/A_{200} ratio was 1.15-1.2.

DNA polymerase assay

Standard assays were done in 50 μl, as previously described (3), but the concentration of [^{3}H]dTTP was 1.25×10^{-6} M (10 Ci/mmol, New England Nuclear) and 5 μg of denatured (24 hr, 0.2 N KOH, 20°C) salmon DNA in 0.01 M Tris (pH 8.0)-1 mM EDTA was included. The final concentrations of KCl and Triton X-100 varied slightly depending on the experiment. Incubation was for 1-2 hr at 38°C. [^{3}H]dTTP incorporated into DNA was precipitated by the addition of 100 μg of yeast RNA and 10 volumes of 5% trichloroacetic acid (TCA). After 10 min at 0°C, the precipitate was poured onto Millipore filters that had been soaked in a saturated solution of pyrophosphate, and washed 4 times with 5% TCA. Radioactivity was counted in a toluene-based scintillation fluid, and background (80 ± 10 cpm/filter) was subtracted.

RESULTS

Solubilization of the virus-associated DNA polymerase

To solubilize the DNA polymerase of either strain, a virus solution in (about) 50% buffered glycerol was incubated for 15 min at 38°C with 3-5 volumes of disruption buffer containing Triton X-100 at a final concentration of 0.5%. After centrifugation (< 1 ml) in a polycarbonate tube at 150,000 × *g* for 15 min, the supernatant contained 75% ± 4% (average of 5 preparations ± SE) of the enzymatic activity of an uncentrifuged solution. Similar experiments with radioactive virus, which contained amino acid-labeled protein (10) or glucosamine-labeled glycoprotein (11), indicated that about 80% ± 5% (average of 4 preparations ± SE) of the viral protein

Abbreviations: RSV, Rous sarcoma virus; RSV-A and RSV-C, the serological subgroups A and C of RSV, respectively; PR RSV, Prague strain; SR RSV, Schmidt-Ruppin strain; DTT, dithiothreitol; BSA, bovine serum albumin.

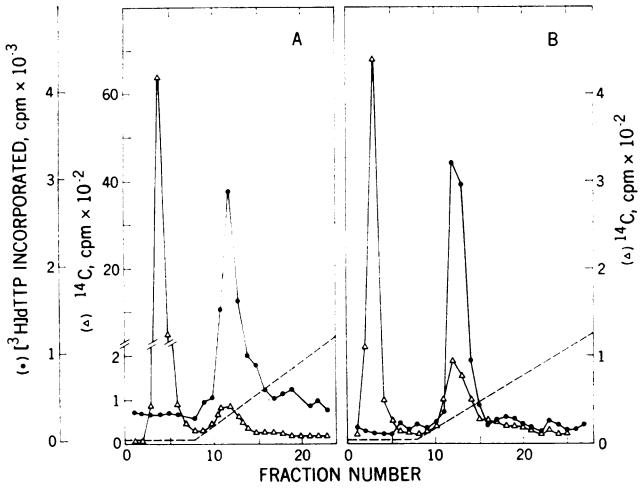

FIG. 1. DEAE-cellulose chromatography of the soluble DNA polymerase derived from [^{14}C]amino acid-labeled (*A*) or [^{14}C]-glucosamine-labeled (*B*) PR RSV-C. (*A*) 30 μl of PR RSV-C (18 A_{260}/ml) and 20 μl of [^{14}C]amino acid-labeled PR RSV-C (about 100,000 cpm), 0.5 ml of disruption buffer containing 0.02 M KCl, and 25 μl of Triton X-100 (10% v/v) were processed and centrifuged (150,000 × *g*) as described for Fig. 2*A*. The supernatant was applied (at 5°C) to a DEAE-cellulose column (3 × 0.6 cm) equilibrated in buffer. After the column was washed with several ml of buffer, a linear KCl gradient in the same buffer was applied and 10-min (about 1-ml) fractions were collected. Analysis of the fractions was as described for Fig. 2*A*. (*B*) Same as for *A*, but 20 μl of [^{14}C]glucosamine-labeled PR RSV-C (about 100,000 cpm) was used. The extremes of the KCl gradient (- - -) represent 0.02 M KCl and 0.6 M KCl.

and the glycoprotein remained in the supernatant. Since under the same conditions of centrifugation untreated virus was pelleted, the enzymatic activity and viral proteins in the supernatant must have been released from intact virus in a soluble form.

Chromatography of the soluble DNA polymerase on DEAE-cellulose

DEAE-cellulose chromatography of the soluble DNA polymerase of [^{14}C]amino acid-labeled virus, corresponding to about 200 μg of viral protein, is shown in Fig. 1*A*. About 30% of the starting enzymatic activity eluted with about 2% (4 μg) of the viral protein at 0.15–0.2 M KCl. Some enzymatic activity eluted at higher KCl concentrations. Since 70% of the starting enzymatic activity was lost, this corresponds to a 15-fold enrichment of the enzyme. Because 80% of the [^{14}C]protein applied to the column was recovered, it may be concluded that the enzyme represents, at most, 2% of the soluble [^{14}C]-proteins of the virus. Fig. 1*B* illustrates that about 30% of the radioactive glycoprotein (11) of solubilized virus that eluted from the column cochromatographed with the viral DNA polymerase. Elution from DEAE-cellulose at 0.15–0.2 M KCl indicates that the enzyme has an isoelectric point below 6 (13), which is similar to that of other nucleic acid polymerases (14) of similar size (see below).

Sedimentation coefficient of the solubilized viral DNA polymerase before and after incubation with RNase

The DNA polymerase sedimented as a single component, with an estimated (15) $s_{20,w}$ of 8 S based on the 4.3 S value of a bovine serum albumin ([^{14}C]BSA) (16) marker (see Fig. 4*A*). After incubation with pancreatic RNase, the $s_{20,w}$ of the enzyme was reduced to 6 S (see Fig. 4*B*). The recovery of DNA polymerase activity after sucrose gradient sedimentation was 80% ± 5% (average of 4 experiments ±SE) without RNase treatment and from 40 to 80% after RNase treatment. The enzyme had a half-life in the gradient solution of ≧ 4 weeks at 5°C.

The molecular weight of the 8S DNA polymerase can be estimated by the formula $s_{w1}/s_{w2} = (MW_1/MW_2)^{2/3}$ (17) to be about 170,000 (BSA standard = 67,000) (16), and that of the 6S DNA polymerase to be about 110,000. The difference between the estimated weights of the 8S and the 6S forms of the DNA polymerase may be due to the removal by RNase of small pieces of enzyme-associated RNA.

Hydrodynamic and pherographic analyses of the 8S and the 6S viral DNA polymerase and other soluble components of the virus

It is shown in Fig. 2*A* that the 8S DNA polymerase did not correspond to a distinct peak of soluble [^{14}C]protein of the virus. The enzymatic activity overlapped with a [^{14}C]protein component that had a slightly higher $s_{20,w}$ than the enzymatic activity and consisted of about 12% of the radioactive protein in the gradient. This component was identified (11) as an "8S" glycoprotein complex; it will be referred to here as 9S component (Fig. 2*B*, *C*) based on a 4.3S BSA standard. (The previous estimate (11) was based on tRNA, assumed to be 4S.) About 85% of the [^{14}C]protein sedimented more slowly than the 8S DNA polymerase, at about 1–2 S (Fig. 2*A*, *C*). Both the $s_{20,w}$ (11) and the electrophoretic properties (not shown, ref. 10) of this material indicated that it was the viral group-specific antigen. The three peak fractions (80%) of the 8S DNA polymerase in Fig. 2*A* cosedimented with about 5% of the soluble [^{14}C]protein, representing a 15-fold purification of the enzyme.

If the $s_{20,w}$ of the DNA polymerase was reduced to 6 S by treatment with RNase (Fig. 2*C*), the sedimentation distribution of the major viral protein components was not significantly altered; however, the three peak fractions (80%) of the enzymatic activity cosedimented with only 2% of the viral [^{14}C]proteins (Fig. 2*C*). This represents a 40-fold purification of the enzyme. Fig. 3*A* shows electrophoresis of the [^{14}C]protein that cosedimented with the 6S DNA polymerase shown in Fig. 2*C*. Unfractionated [^{3}H]amino acid-labeled virus was added to provide electrophoretic markers for the major structural proteins of the virus, which included the proteins of the group-specific antigen gs_1, gs_2, and gs_3, and the two viral glycoproteins, I (37,000 daltons) and II (105,000 daltons) (11). The majority of the [^{14}C]protein had the same electrophoretic mobility as the glycoproteins, II and I (see Fig. 3*B*) (11); very little ^{14}C coelectrophoresed with the components of the group-specific antigen. In addition, at least one new ^{14}C peak appeared in the gel, labeled *X* (Fig. 3*A*); it had not been identified previously (10, 11). A very similar electrophoretic pattern was obtained when the [^{14}C]protein that cochromatographed with the enzyme on DEAE-cellulose was analyzed under these conditions (not shown). It can be deduced from these experiments that the DNA polymerase is not identical with the group-specific antigen of the virus.

Cosedimentation of the 8S DNA polymerase and the soluble [^{14}C]glycoprotein of the virus is shown in Fig. 2*B*. The 8S polymerase overlapped with 25% of the 9S glycoprotein com-

plex, whereas the 6S DNA polymerase overlapped with only 10% of the soluble [^{14}C]glycoprotein (Fig. 2*D*). Sedimentation of the enzymatic activity after overnight precipitation in 60% ammonium sulfate in the presence of 50 μg of BSA did not change the relative sedimentation distributions of the enzyme and the glycoprotein (not shown).

Electrophoresis of the [^{14}C]glycoprotein that cosedimented with the 6S DNA polymerase is shown in Fig. 3*B*. More than 90% of this [^{14}C]glycoprotein coelectrophoresed with the known glycoproteins I and II of unfractionated virus (11); no new distinct glycoprotein component appeared. The same electrophoretic pattern was obtained when the peak fractions

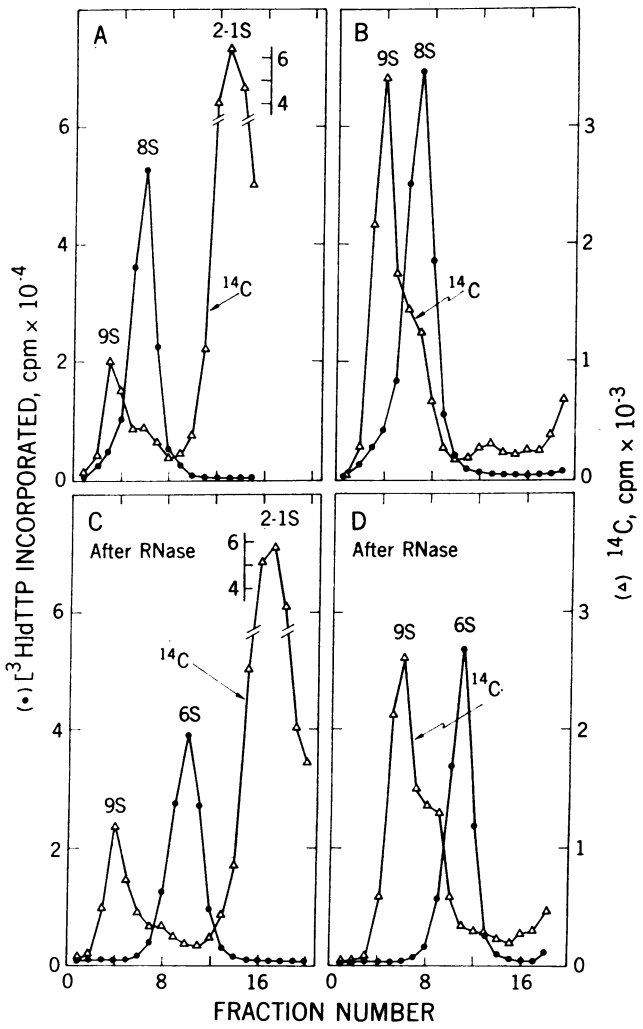

FIG. 2. Sucrose gradient sedimentation of the soluble DNA polymerase of PR RSV-C before (*A*, *B*) and after (*C*, *D*) RNase treatment. (*A*) 750,000 cpm of [^{14}C]amino acid-labeled PR RSV-C, 30 μl of PR RSV-C in 50% buffered glycerol at a concentration of 18 A_{260}/ml, 200 μl of disruption buffer, and 15 μl of Triton X-100 (10% v/v) were incubated at 38°C for 15 min, then centrifuged in a Spinco 40 rotor at 40,000 rpm (20°C) for 15 min in a polycarbonate tube. The supernatant was layered on a linear sucrose gradient (15–30%) containing 0.1 M KCl–0.01 M Tris-(pH 8.0)–1 mM EDTA 0.2 mM DTT–0.2% Triton X-100. The gradient was poured over a 0.3- to 0.4-ml cushion of 65% sucrose in the same buffer. Centrifugation was in a polyallomer tube in a Spinco SW65 rotor at 20°C for 9 hr at 65,000 rpm. 12-drop fractions were collected. 10-μl aliquots were assayed for polymerase activity during a 90-min incubation period. ^{14}C was determined by placing appropriate aliquots in toluene-based scintillation fluid containing 20% NCS (Nuclear-Chicago), and counting in a Tri-Carb scintillation counter. (*B*) [^{14}C]-glucosamine-labeled PR RSV-C containing about 250,000 cpm was mixed with 30 μl of PR RSV-C (18 A_{260}/ml) and processed as described for *A*. (*C*) Same as *A*, but 1 μg of pancreatic RNase (heated at 100°C for 5 min) was included in the disruption mixture. (*D*) Same as *B*, but disrupted in the presence of pancreatic RNase as described for *C* and centrifugation was only for 7 hr.

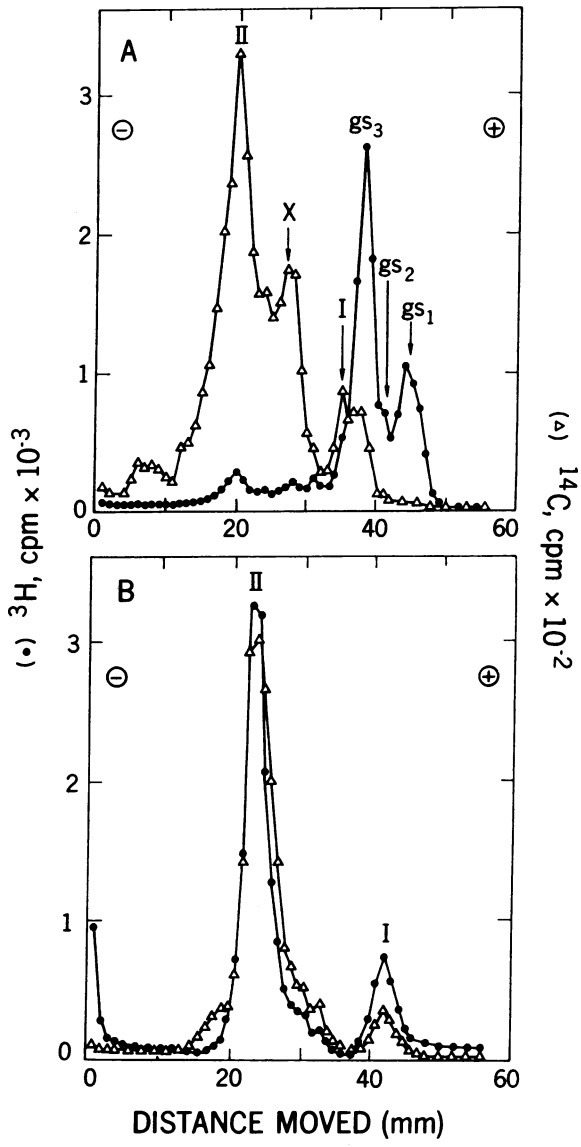

FIG. 3. Electrophoretic analysis of the [^{14}C]amino acid- or [^{14}C]glucosamine-labeled material of solubilized virus that cosediments with the viral DNA polymerase. (*A*) Aliquots of fractions 9–11 (Fig. 2*C*) of [^{14}C]amino acid-labeled virus were precipitated with 5 vol of ethanol and 50 μg of BSA. The precipitate was mixed with an appropriate amount of unfractionated [^{3}H]-amino acid-labeled virus to provide electrophoretic markers and analyzed by electrophoresis in 5% sodium dodecyl sulfate–polyacrylamide gels as described (11). (*B*) Aliquots of fractions 10–12 (Fig. 2*D*) were precipitated as described for *A*. Prior to electrophoresis, unfractionated [^{3}H]glucosamine-labeled PR RSV-C was added to provide glycoprotein reference markers (11).

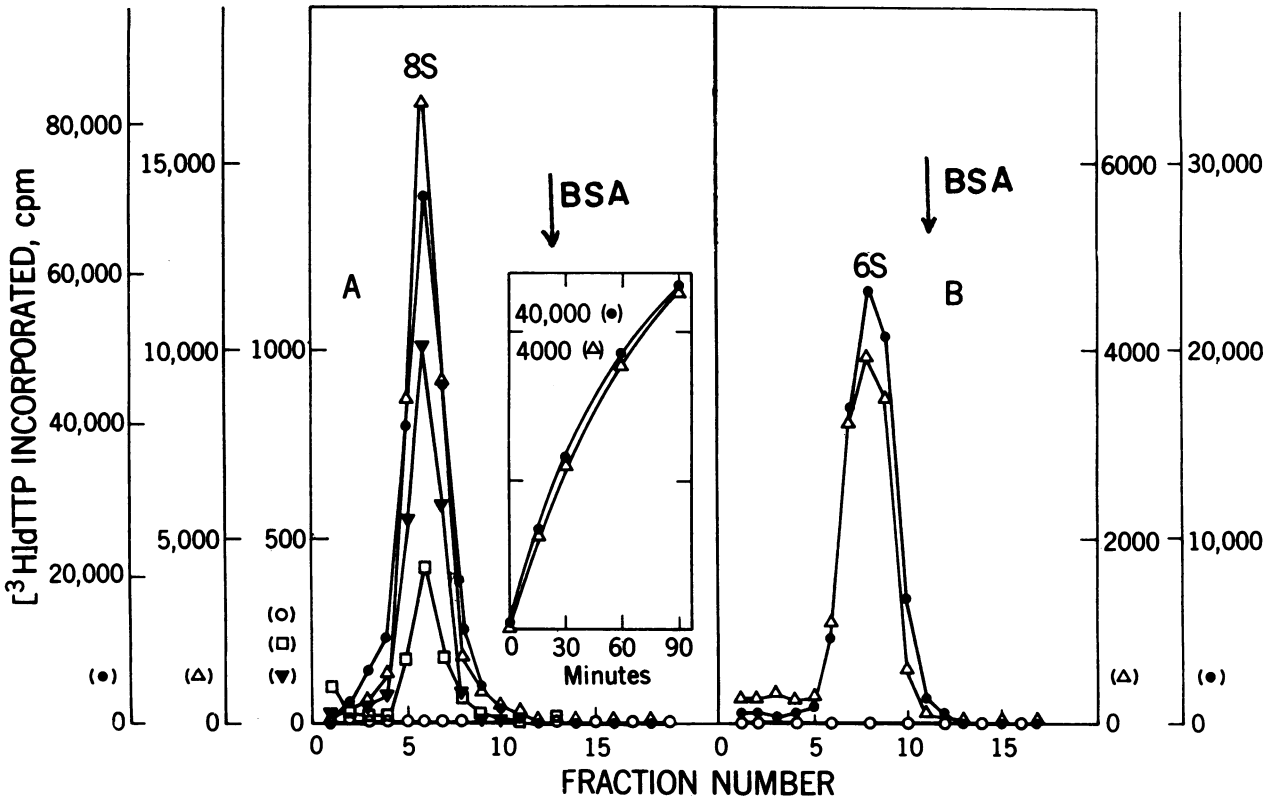

FIG. 4. Analysis of the template activities of various nucleic acids for the 8S (*A*) and the 6S (*B*) viral DNA polymerase activity. Sucrose gradient sedimentation of the soluble DNA polymerase was as described for Fig. 2*A*, *B*. The [^{14}C]BSA, noted by an *arrow*, was present as a marker. (*A*) 10-μl aliquots of each fraction were incubated for 90 min with either 5 μg of salmon DNA (●—●) or 2.5 μg of 60–70S PR RSV-C RNA, (△—△), or 2.5 μg of heat-dissociated (12, 18) 60–70S PR RSV RNA (▼—▼), or 2 μg of tobacco mosaic virus RNA (□—□), or no added nucleic acid (○—○) in the standard DNA polymerase assay. Three μg of influenza virus RNA had essentially the same template activity as 2.5 μg of heat-dissociated RSV RNA (not shown). Preparation of 60–70S PR RSV RNA: PR RSV-C RNA (12, 18) was incubated in 250 μl of 0.01 M Tris (pH 7.4)–0.15 M NaCl–2 mM $MgCl_2$ that contained 10 μg of DNase (RNase-free) for 15 min at 38°C and 1 hr at room temperature to degrade associated DNA (22). It was then diluted with 1 ml of standard buffer containing 0.2% sodium dodecyl sulfate, extracted once with phenol, and, after ethanol precipitation, the 60–70S PR RSV-C RNA was prepared by sucrose gradient sedimentation (12). The insert shows the rate of [^{3}H]dTTP incorporation by 10-μl aliquots of fraction 6 in the presence of 60–70S PR RSV RNA or salmon DNA. (*B*) RNase treatment was as described for Fig. 2. The respective fractions were tested as described for *A*.

of the 9S [^{14}C]glycoprotein component that did not sediment with enzymatic activity were analyzed (not shown), or when the [^{14}C]glycoprotein that cochromatographed with the enzyme on DEAE-cellulose (Fig. 2*A*) was electrophoresed (not shown). Results very similar to those described for PR RSV-C were obtained with SR RSV-A.

It may be concluded that the 6S DNA polymerase differs from 90% of the viral glycoprotein. The 10% of the viral glycoprotein that could not be separated from the 6S DNA polymerase by our methods had the same electrophoretic distribution as the two known glycoproteins of the virus.

Template specificity of the soluble DNA polymerase

The soluble DNA polymerase presented an opportunity to examine whether the enzyme was able to use added RNA templates, and whether it preferred 60–70S PR RSV RNA over other RNAs. This question could not be answered previously because the enzyme had not been solubilized, nor was it experimentally possible to eliminate the endogenous RNA template without at the same time affecting added natural RNA templates.

The 60–70S RNA of PR RSV-C was found in different experiments to have, per unit weight (saturation curves were not determined), 10–40% of the template activity of salmon DNA for both the 8S and the 6S DNA polymerase (Fig. 4*A*, *B*). The rate with the peak fraction of enzyme for native RSV RNA- and salmon DNA-primed enzymatic activities of the 8S DNA polymerase were indistinguishable (Fig. 4*A*). Heat-dissociated 60–70S PR RSV RNA (12, 18) had only 2–6% (range of four experiments) of the template activity of native 60–70S RNA (Fig. 4*A*). Other natural RNAs like tobacco mosaic virus RNA had 2%, and influenza virus RNA 5%, of the template activity of 60–70S PR RSV RNA for the 8S DNA polymerase (Fig. 4*A*).

Both the RNA- and the DNA-primed polymerase activities of the 8S and the 6S form of the enzyme coincided in the gradients shown in Fig. 4. Neither the 8S nor the 6S DNA polymerase had detectable endogenous template activities. Further, it was found when different fractions were mixed that the RNA-primed enzymatic activities of all fractions of the 8S DNA polymerase peak tested were 70–100% additive; the same was true when DNA-primed activities were

measured. This suggests that no enhancing or inhibiting factor was present in this region of the gradient.

DISCUSSION

The distinctive RNase-sensitivity of the $s_{20,w}$ of the viral DNA-polymerase permitted a 40-fold purification of the enzyme based on the soluble radioactive protein of the virus. Since the particle weight of RSV is about 5×10^8, and its protein content is 60% (19), 1.5% at most of the mass of the virion can be DNA polymerase. This calculation assumes that the specific radioactivities of the enzyme and the structural proteins of the virus are the same. It follows that a maximum of 70 6S DNA polymerase molecules (110,000 daltons) may be present per virion. However, this is considered to be an upper estimate, because the 40-fold purified enzyme was contaminated by other structural proteins of the virus. The purified enzyme contained at least one protein component that had not previously been identified (10, 11) as a structural component of the virus. The enzyme also contained 10% of the soluble viral glycoprotein, which may not be an essential part of the polymerase because it consisted mainly of the two components of the 9S glycoprotein complex of the virus; the complex had no enzymatic activity.

Neither the 8S and the 6S DNA polymerase had endogenous template activities. This leaves open the question whether the reduction of the $s_{20,w}$ of the enzyme from 8S to 6S by RNase treatment is due to removal of short pieces of viral, or perhaps of cellular, RNA (20) without template activity. Such short pieces of viral RNA presumably arise by degradation of the 60–70S viral RNA (20) by virus-associated RNase (21), as tumor virus RNA is known to become RNase-sensitive in the presence of nonionic detergents (20). Short pieces of enzyme-associated RNA might enhance the activity of templates added to the enzyme. This is compatible with the decrease of enzymatic activity observed after RNase treatment.

The coincidence in sucrose gradients of both the RSV RNA- and the DNA-primed DNA polymerase activities, in both the 8S and the 6S form, makes it likely that the two activities reside in the same enzyme. This evidence does not exclude, however, that different sites on the enzyme or different factors might specify the RNA- or DNA-dependent activities. It would appear, then, that this enzyme, which is known to have rather nonspecific template requirements if primed by natural DNAs (4–8) or synthetic nucleic acids (4, 9), has specific template requirements for natural RNAs, i.e., preferential affinity for 60–70S RSV RNA. The finding that heat dissociation reduces the template activity of 60–70S RSV RNA to that of other natural RNAs suggests that the peculiar secondary or subunit structure of 60–70S RSV RNA (12, 18) may be essential for its template activity for the viral DNA polymerase.

NOTE ADDED IN PROOF

The soluble DNA polymerase was separated from more than 99% of the viral glycoprotein by chromatography on phosphocellulose.

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