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Purification and Properties of Spleen Necrosis Virus DNA Polymerase

SATOSHI MIZUTANI AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

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DNA polymerase was purified to apparent electrophoretic homogeneity from virions of spleen necrosis virus (SNV). (SNV is a member of the reticuloendotheliosis group of avian ribodeoxyviruses.) The SNV DNA polymerase appears to consist of a single polypeptide with a molecular weight of 68,000. The SNV DNA polymerase has a preference for Mn^{2+} for DNA synthesis with an RNA template and Mg^{2+} for DNA synthesis with a deoxyribohomopolymer template. At the optimum concentrations of divalent cation, the relative rates of DNA synthesis by SNV DNA polymerase with different template·primers were similar to the relative rates of DNA synthesis by an avian leukosis virus DNA polymerase, with the exception of a lower relative rate of DNA synthesis by SNV DNA polymerase with SNV RNA. However, in contrast to DNA synthesized by the avian leukosis virus DNA polymerase with an SNV RNA template, DNA synthesized by SNV DNA polymerase with an SNV RNA template did not hybridize to the SNV RNA. SNV DNA polymerase has RNase H activity which is antigenically distinct from the RNase H activity of avian leukosis-sarcoma virus DNA polymerase.

The reticuloendotheliosis group of avian ribodeoxyviruses (REV) includes spleen necrosis virus (SNV), reticuloendotheliosis virus (strain T), duck infectious anemia virus, and chick syncytial virus (20). Like the replication of the other ribodeoxyviruses, the replication of the REV involves a DNA provirus integrated with host cell DNA (2, 9).

However, in contrast to most other ribodeoxyviruses, REV virions have very low endogenous RNA-directed DNA polymerase activity in the presence of Mg^{2+} (15a). Addition of $(dT)_{12-18}$ or $(dC)_{12-18}$ as primers to the REV endogenous DNA polymerase reaction mixture only stimulated [^{3}H]dTMP incorporation by REV virions 20 to 50% over the reactions without these oligomers (unpublished data). Furthermore, REV virions contain an RNA polymerase activity distinct from the DNA polymerase activity (15a; S. Mizutani and H. M. Temin, manuscript in preparation). Therefore, the enzymatic machinery involved in the synthesis of REV DNA seems to be different from that of most other ribodeoxyviruses.

The REV DNA polymerases are closely related to each other by serological tests. In addition, the DNA polymerases of reticuloendotheliosis viruses, avian leukosis-sarcoma viruses, and fowl cells have class-specific serological relationships (15).

In the present study, the DNA polymerase of SNV was purified to homogeneity and was characterized as to size, cation requirements, template specificities, and presence of RNase H activity.

MATERIALS AND METHODS

Viruses. SNV and Carr-Zilber-associated virus (CZAV) (an avian leukosis virus [ALV]) were grown and purified by the methods described previously (14, 15).

Antibodies. Antibodies against SNV DNA polymerase and against avian myeloblastosis virus (AMV) DNA polymerase were described previously (14, 15).

S1 nuclease. S1 nuclease was prepared from N. P. Sanzyme (Enzyme Development Corporation, New York, N.Y.) according to the method of Vogt (24).

Purification of DNA polymerases. (i) Solubilization of DNA polymerases from virions. Purified virions (15 to 20 mg of protein) of SNV or CZAV were suspended in 0.02 M Tris-hydrochloride (pH 8.0) with 0.001 M EDTA, 0.5 M NaCl, 0.5% Triton X-100, and 0.005 M dithiothreitol at a concentration of 2 mg of virus protein per ml. The virus suspensions were kept in an ice bath for 15 min and then were centrifuged at 105,000 × *g* for 1 h at 4 C. The supernatants were used for the next step of the purification.

(ii) Bio Gel A column chromatography. Bio Gel A, 0.5-m (Bio-Rad) columns (2.5 by 60 cm), were equilibrated with 0.01 M Tris-hydrochloride (pH 8.0)

containing 0.5 M NaCl, 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 0.2% Triton X-100, and 10% glycerol. The supernatants of the disrupted virus extracts were applied to the columns and were fractionated with the same buffer. DNA polymerase activity was assayed in each fraction by the method described later. Fractions containing DNA polymerase activity were pooled and dialyzed against buffer A (0.02 M Tris-hydrochloride, pH 8.0, containing 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 0.2% Triton X-100, and 30% glycerol) before the next column chromatography.

(iii) Phosphocellulose column chromatography. Phosphocellulose (P-11, Whatman) columns (0.9 by 8 cm) were equilibrated with buffer A. The pools of fractions with DNA polymerase activity from the Bio Gel A columns were absorbed to the phosphocellulose columns, and the columns were washed with 2 bed volumes of buffer A. The DNA polymerases were then eluted with 100 ml of an NaCl gradient (0 to 0.5 M) in buffer A. Fractions containing DNA polymerase activity were pooled and dialyzed against buffer B (0.02 M Tris-hydrochloride, pH 7.5, containing 0.005 M 2-mercaptoethanol, 0.001 M EDTA, 0.2% Triton X-100, and 30% glycerol).

(iv) DEAE-Bio Gel A column chromatography. DEAE-Bio Gel A (Bio-Rad) columns (0.9 by 8 cm) were equilibrated with buffer B. The pools of fractions with DNA polymerase activity from the phosphocellulose columns were adsorbed to the DEAE-Bio Gel A columns, and DNA polymerase was eluted with 100 ml of an NaCl gradient (0 to 0.5 M) in buffer B. Each fraction (25 μl) was assayed for DNA polymerase and RNase activities by the methods described later.

(v) Concentration of DNA polymerase. Fractions from the DEAE-Bio Gel column chromatography which contained DNA polymerase activity and no RNase activity were pooled, diluted with three volumes of buffer A, and adsorbed to small phosphocellulose P-11 columns (2-ml bed volume). After washing the columns with 5 bed volumes of buffer A, DNA polymerases were eluted with 0.5 M NaCl in buffer A containing 10% glycerol. The fractions containing DNA polymerase activity were pooled and dialyzed against buffer A containing 2 mM $MgCl_2$ and 50% glycerol.

(vi) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The method described by Laemmli (11) was followed using 7.5% acrylamide. With this method 0.05 μg of bovine serum albumin could be seen as a band after staining with Coomassie brilliant blue.

(vii) DNA polymerase assay. Unless otherwise indicated, a standard DNA polymerase assay was used (15). A 12.5-μg amount of activated calf thymus DNA, 3 μg of SNV RNA, or 0.005 absorbancy unit at 260 nm of $(rA)_n \cdot (dT)_{12-18}$, $(dA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, or $(dC)_n \cdot (dG)_{12-18}$ per reaction were used as template·primers. (The optimum concentrations of divalent cations were used for each template·primer and are indicated in the text.) When SNV RNA (the total virion RNA) was used as a template·primer, actinomycin D (100 μg/ml) was included in the reaction. The volume of DNA polymerase used per reaction was 25 μl. The protein concentration of the DNA polymerase is indicated for each reaction. [^{3}H]dTTP (5,000 counts/min per pmol) was used in most of the reactions. [^{3}H]dGTP (4,250 counts/min per pmol) was used for the $(rC)_n \cdot (dG)_{12-18}$- or $(dC)_n \cdot (dG)_{12-18}$-directed reactions. With all template·primers, the reactions proceeded linearly for 20 min and then gradually slowed down.

(viii) RNase and DNase activities. Chicken [^{3}H]RNA (2 μg, 1,000 counts/min per μg) and chicken [^{3}H]DNA (2 μg, 1,300 counts/min per μg) prepared from chicken fibroblast cells in culture were incubated at 37 C for 1 h in 50 μl of 0.02 M Tris-hydrochloride (pH 7.5) with 20 mM $MgCl_2$ with or without the same amount of DNA polymerase used in the DNA polymerase assays. The entire reaction mixtures were put on filter papers (Whatman no. 541, 2.5-cm diameter) which had been pretreated with 25 μl of 0.1 M sodium pyrophosphate, and the filters were washed with trichloroacetic acid in the same manner as in the DNA polymerase assay. Under these conditions, 1 μg of pancreatic RNase per ml or 1 μg of pancreatic DNase per ml digested over 95% of the added RNA or DNA, respectively.

(ix) RNase H assay. Substrate preparation: [^{3}H]RNA-DNA was prepared with *Escherichia coli* RNA polymerase (a kind gift of R. R. Burgess) and heat-denatured calf thymus DNA. The reaction mixture contained 100 μM each rATP, rCTP, and rGTP, 2.3 μM [^{3}H]uridine 5'-triphosphate (22 Ci/mmol), 10 mM $MgCl_2$, 0.1 M KCl, 0.04 M Tris-hydrochloride (pH 8.0), 100 μg of heat-denatured calf thymus DNA per ml, and 2 μg of *E. coli* RNA polymerase in a total volume of 0.2 ml. The reaction mixture was incubated at 37 C for 1 h and was extracted three times with an equal volume of a chloroform-isoamyl alcohol mixture (24:1), and the product was precipitated with two volumes of absolute alcohol at −20 C overnight. The precipitate was collected by centrifugation and dissolved in 0.5 ml of 0.01 M Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl and 0.001 M EDTA. The solution was applied to a Sephadex G-75 column (0.5 by 40 cm). The fractions at the void volume containing radioactivity were pooled, and the product was precipitated with two volumes of absolute alcohol at −20 C overnight. The precipitate was collected by centrifugation and dissolved in 0.2 ml of 0.01 M Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl and 0.001 M EDTA. The product was treated with S1 nuclease as described later, extracted with the chloroform-isoamyl alcohol mixture, and precipitated with ethanol as described above. Eighty percent of the trichloroacetic acid-insoluble radioactivity was recovered after S1 nuclease treatment.

[^{3}H]$(rA)_n \cdot (dT)_m$ was made by mixing [^{3}H]$(rA)_n$ (Miles Laboratories, 85.6 μCi/μmol) and a 1.5-fold excess in nucleotide concentration of $(dT)_m$.

The assay was carried out in 0.05 ml of solution containing 20 mM Tris-hydrochloride (pH 8.0), 2 mM $MgCl_2$, 5 mM dithiothreitol, and the indicated amounts of [^{3}H]RNA·DNA or [^{3}H]$(rA)_n \cdot (dT)_m$ and DNA polymerase.

The reaction mixtures were incubated at 37 C, and the reactions were stopped by adding 500 μg of bovine

serum albumin and 5 ml of 10% trichloroacetic acid. After chilling in an ice bath for 15 min, the precipitates were collected by centrifugation and were resuspended in 5 ml of ice cold 10% trichloroacetic acid. The precipitates were collected by centrifugation, dissolved in 0.6 ml of 0.2 N NaOH, and then neutralized with 0.24 ml of 0.5 N HCl. Each solution was transferred into a scintillation vial, and the radioactivity was counted with 10 ml of Scintisol scintillation fluid.

(x) Protein assay. Interfering materials such as dithiothreitol, mercaptoethanol, Tris, glycerol, and Triton X-100 made it impossible to use the Lowry method for protein determination in the DNA polymerase preparations. The micromethod of Nakao et al. (19) was therefore used.

Nucleic acid hybridization. (i) Purification of SNV RNA. Purified virions were suspended at a concentration of 2 mg of viral protein per ml in 0.05 M sodium acetate buffer (pH 5.1) containing 0.1 M NaCl, 0.1% Triton X-100, 0.5% SDS, and 10 mg of bentonite per ml. The suspension was extracted two times with equal volumes of phenol-cresol mixture (10). The aqueous phase was adjusted to pH 7.0 with NaOH and then was extracted twice with equal volumes of chloroform-isoamyl alcohol mixture (24:1). Two volumes of ethanol and 0.1 volume of 20% potassium acetate solution were added to the final aqueous phase, and the mixture was kept at −20 C overnight. The precipitate was collected by centrifugation and dissolved in 0.01 M Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl and 0.001 M EDTA (STE buffer). The solution was treated with DNase I (RNase-free grade, Worthington) at 50 μg/ml in the presence of 10 mM $MgCl_2$ at 37 C for 30 min. The solution was extracted with the chloroform-isoamyl alcohol mixture three times, and RNA was precipitated with ethanol and potassium acetate as described above. RNA was collected by centrifugation and was dissolved in STE buffer. With this method about 17 μg of RNA was recovered from 1 mg of protein of SNV particles.

(ii) Preparation of DNA copy of SNV RNA. DNA synthesis was carried out in the standard DNA polymerase reaction with 2 mM $MgCl_2$ or 2 mM $MnCl_2$ in the presence of actinomycin D (100 μg/ml) at 37 C for 60 min utilizing SNV RNA and purified SNV DNA polymerase or purified CZAV DNA polymerase.

The reaction mixtures were extracted with equal volumes of chloroform-isoamyl alcohol (24:1), and then [^{3}H]DNA was precipitated with two volumes of ethanol at −20 C overnight. The precipitate was dissolved in 0.5 ml of STE buffer and applied to a Sephadex G-75 column (0.5 by 40 cm). The fractions containing radioactivity at the void volume were pooled and precipitated with ethanol at −20 C overnight. The precipitates were dissolved in 50 μl of STE buffer. Just before nucleic acid hybridization the required amount of [^{3}H]DNA was treated with 0.5 N NaOH at 37 C for 30 min in a plastic tube. After neutralization with HCl, the [^{3}H]DNA was used for nucleic acid hybridization. Before the alkali treatment, approximately 95% of the [^{3}H]DNA was resistant to S1 nuclease digestion. After the treatment, the [^{3}H]DNA became almost 100% sensitive to S1 nuclease digestion.

Nucleic acid hybridization. All nucleic acid hybridizations were carried out in volumes of 25 μl sealed in glass 25-μl micropipettes (Yankee disposable pipettes, Clay Adams Co., N.J.). The hybridization mixtures contained 0.6 M NaCl, 0.001 M EDTA, 0.05 M Tris-hydrochloride (pH 7.5), 0.01% SDS, 1% phenol, and 7.5 mg of yeast RNA per ml. Annealing was carried out at 63 C. The extent of hybridization was determined by digestion with S1 nuclease. Samples were diluted to a final volume of 250 μl in digestion buffer containing 0.03 M Na-acetate buffer (pH 4.3), 0.0018 M $ZnCl_2$, 0.3 M NaCl, 200 μg of denatured calf thymus DNA per ml, and excess S1 nuclease. The mixtures were incubated for 60 min at 37 C (7). RNA and DNA concentrations in the hybridization mixtures were 1.14×10^{-4} M and 0.8×10^{-7} to 3.2×10^{-7} M, respectively. After S1 nuclease digestion, the trichloroacetic acid-insoluble counts were expressed as percent hybridization of input [^{3}H]DNA counts.

RESULTS

Purification of SNV DNA polymerase. Purified virions of SNV were disrupted with 0.5% Triton X-100, and the extract was subjected to the column chromatography steps listed in Table 1. The overall recovery of protein was 0.12%, and the recovery of DNA polymerase

TABLE 1. *Purification of SNV DNA polymerase*[a]

Purification step	Total protein (mg)	Activated calf thymus DNA			$(rA)_n \cdot (dT)_{12-18}$		
		Sp act (nmol/mg of protein/10 min)	Total activity (nmol/10 min)	Recovery (%)	Sp act (nmol/mg of protein/10 min)	Total activity (nmol/10 min)	Recovery (%)
Extract	16.20	7.5	121	(100)	24	390	(100)
Bio Gel A	1.27	13.5	17.1	14.1	36	45.7	11.7
Phosphocellulose	0.11	152.0	16.7	13.8	346	38.1	9.8
DEAE-Bio Gel A	0.02	605.0	12.1	10.0	3960	79.2	20.3

[a] A 16.20-mg amount of protein of purified SNV virions was treated by the techniques listed (see Materials and Methods). At each step a portion was assayed for DNA polymerase activity with either activated calf thymus DNA or $(rA)_n \cdot (dT)_{12-18}$ template·primers as described in the text.

activity was 10% with activated calf thymus DNA and 20% with $(rA)_n \cdot (dT)_{12-18}$ as template·primers for DNA polymerase assay. After purification, the specific activity of the SNV DNA polymerase was increased by about 80-fold with activated calf thymus DNA template·primer and by 165-fold with $(rA)_n \cdot (dT)_{12-18}$ template·primer.

To determine the extent of contamination by other proteins of the SNV DNA polymerase preparation, SDS-polyacrylamide gel electrophoresis was carried out (Fig. 1). A single band was observed when up to 2 μg of SNV DNA polymerase was run on an SDS-polyacrylamide gel. Under the conditions used for SDS-polyacrylamide gel electrophoresis, about 0.05 μg of bovine serum albumin could be seen as a band after staining with Coomassie brilliant blue. Therefore, the preparation of SNV DNA polymerase seems to be relatively uncontaminated and to contain only a single polypeptide.

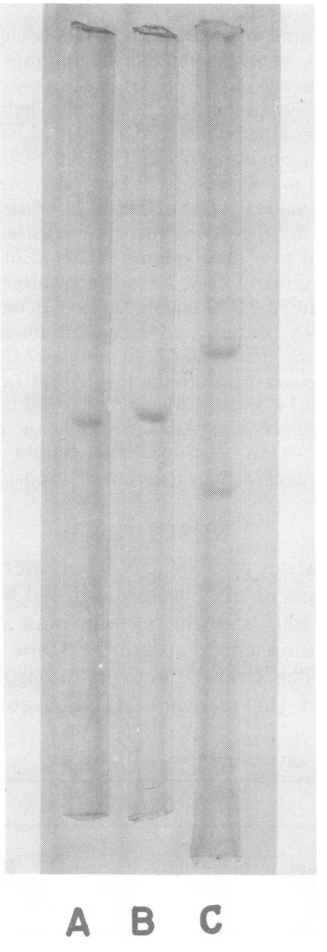

FIG. 1. *SDS-polyacrylamide gel electrophoresis of SNV and CZAV DNA polymerases. Migration began at the top edge of the gels, and the fronts of migration were 10 cm from the origins. (A) Bovine serum albumin, 1 μg; (B) SNV DNA polymerase, 1 μg; (C) CZAV DNA polymerase, 2 μg. The molecular weight of bovine serum albumin is 68,000.*

The molecular weight of the SNV DNA polymerase polypeptide was estimated about 68,000 from the relative distance of migration in SDS-gels of the following standard proteins: chymotrypsinogen A, ovalbumin, bovine serum albumin, and phospholipase C. The molecular weight of 68,000 roughly agrees with the previous estimate of 70,000 from the results of gel filtration under nondenaturing conditions (15) and indicates that the SNV DNA polymerase consists of one copy of a single polypeptide. In contrast, an SDS-polyacrylamide gel electropherogram of the purified DNA polymerase isolated from virions of CZAV showed two bands (Fig. 1) like the DNA polymerases of other avian leukosis-sarcoma viruses (8).

Ribonuclease and deoxyribonuclease activities in the purified SNV DNA polymerase preparations were examined by the methods described above. Only DNA polymerase preparations that made less than 5% of the [^{3}H]RNA and [^{3}H]DNA acid soluble were used in the present study. All DNA polymerase preparations used in the present study were also free (less than 1% of input radioactivity made acid soluble) of nuclease activities against [^{3}H]$(rA)_n$ and [^{3}H]$(dC\text{-}dI)_n$.

Utilization of template·primers by the purified SNV DNA polymerase. Requirements for SNV DNA polymerase activity are shown in Table 2. The maximum DNA polymerase activity in the presence of $MgCl_2$ and activated calf thymus DNA as a template·primer was observed in the presence of four deoxyribonucleoside triphosphates. However, the omission of any one deoxyribonucleoside triphosphate still allowed 40 to 45% of the DNA polymerase activity. $(dT)_{12-18}$ or $(dC)_{12-18}$ alone could not initiate polymerization of dTMP. These results indicate that the dTMP incorporation was the result of DNA polymerase activity rather than terminal transferase activity.

The optimum divalent cation concentrations for several different template·primers were examined (Fig. 2 and 3). The optimum $MgCl_2$ concentrations were 10 mM for activated calf thymus DNA (Fig. 2A); about 2 mM for SNV RNA (Fig. 2B); and 1 mM for $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, and $(dC)_n \cdot (dG)_{12-18}$ (Fig. 3A, B and C). The optimum $MnCl_2$ concentrations were 2 to 10 mM for activated DNA (Fig. 2A); 2 mM for SNV RNA (Fig. 2B); 0.5 mM for

TABLE 2. *Requirements for SNV DNA polymerase activity*[a]

Mixture	[^{3}H]dTMP incorporated (pmol)	% Activity
Complete	3.40	100
-dATP	1.52	45
-dCTP	1.50	44
-dGTP	1.31	39
-dATP, -dCTP, -GTP	0.77	23
-dATP, -dCTP, -dGTP -Activated DNA +$(dT)_{12-18}$	0	0
-dATP, -dCTP, -dGTP -Activated DNA +$(dC)_{12-18}$	0	0

[a] A 0.05-μg amount of purified SNV DNA polymerase was incubated for 10 min at 37 C in the complete mixture containing 10 mM $MgCl_2$ and activated calf thymus DNA described in Materials and Methods or with the deletions listed.

$(rA)_n \cdot (dT)_{12-18}$ and $(rC)_n \cdot (dG)_{12-18}$ (Fig. 3A and 3B); and 1 mM for $(dC)_n \cdot (dG)_{12-18}$ (Fig. 3C). $(dA)_n \cdot (dT)_{12-18}$ was very poorly utilized as a template·primer by SNV DNA polymerase in the presence of 1 mM $MgCl_2$ (data not shown). $(dA\text{-}dT)_n$ was utilized very well as a template·primer by SNV DNA polymerase with an optimum of 15 mM $MgCl_2$ (data not shown). A preference of SNV DNA polymerase was observed for Mn^{2+} as a divalent cation for the DNA polymerase reaction with an RNA template and for Mg^{2+} for the reaction with a deoxyribohomopolymer template.

The optimum sodium chloride concentrations were determined for the SNV DNA polymerase reactions with $(rA)_n \cdot (dT)_{12-18}$ and activated calf thymus DNA template·primers. With the $(rA)_n \cdot (dT)_{12-18}$ the optimum sodium chloride concentration was 80 mM. There was little effect of different sodium chloride concentrations with the calf thymus DNA template·primer (data not shown).

Using the optimum salt conditions for each template·primer, the rates of DNA synthesis by SNV DNA polymerase with different template·primers were determined. An avian leukosis-sarcoma virus, CZAV, DNA polymerase was used for comparison (Table 3). The rate of DNA synthesis by freshly prepared SNV DNA polymerase with SNV RNA in the presence of $MnCl_2$ was similar to the rate by CZAV DNA polymerase. However, the rate of DNA synthesis by SNV DNA polymerase with SNV RNA as a template·primer diminishes after a few days of storage in 50% glycerol at -20 C. The overall rates of DNA synthesis by SNV DNA polymerase were higher with activated DNA or synthetic homopolymers than the rates of DNA synthesis by CZAV DNA polymerase with corresponding template·primers. The rate of DNA synthesis by SNV DNA polymerase with SNV RNA template·primer in the presence of actinomycin D was low compared to the rate of DNA synthesis with activated calf thymus DNA template·primer. Addition of $(dT)_{12-18}$ (0.6 μg) to the SNV RNA stimulated by about 20% the incorporation of [^{3}H]dTMP by SNV DNA polymerase (data not shown). The rate of DNA synthesis with SNV RNA tem-

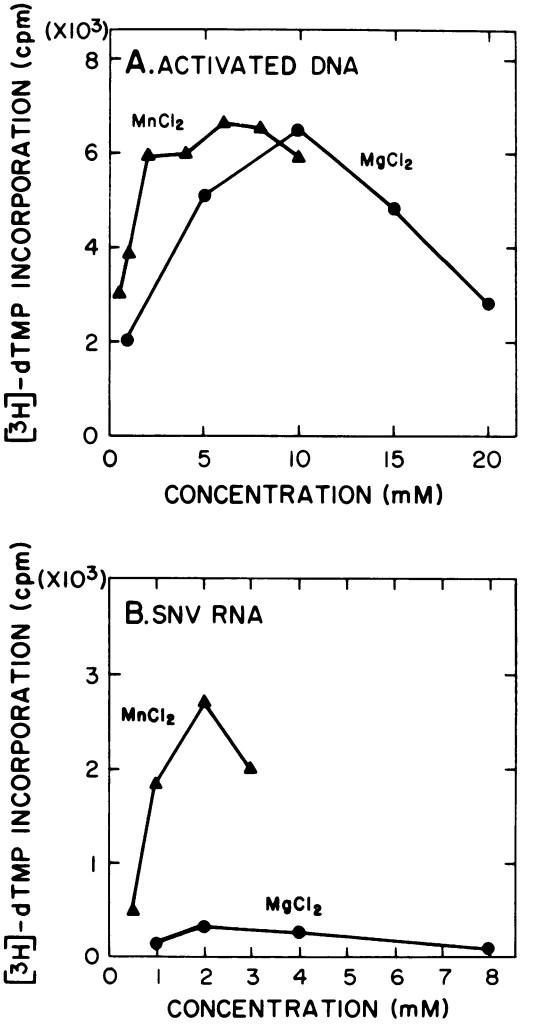

FIG. 2. *Optimum divalent cation concentrations for heteropolymers. Standard DNA polymerase reactions using 0.05 μg of purified SNV DNA polymerase were carried out at 37 C for 10 min with the indicated template·primers and the indicated concentrations of $MgCl_2$ (●) or $MnCl_2$ (▲). (Reactions were linear for over 20 min.)*

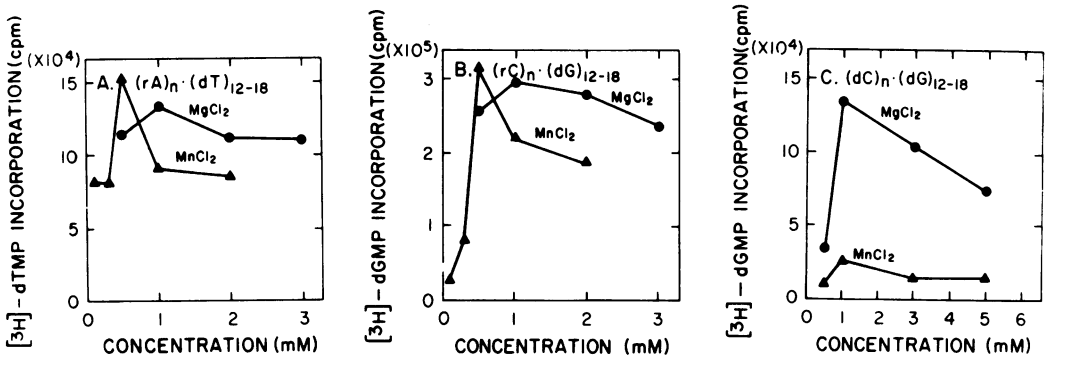

FIG. 3. *Optimum divalent cation concentrations for homopolymers. Conditions were as described in the legend to Fig. 2.*

TABLE 3. *Relative rates of synthesis with different template·primers*[a]

Template·primer	Rate of synthesis (nmol/mg of protein/10 min)	
	SNV DNA polymerase	CZAV DNA polymerase
(a) SNV RNA[b]	25	35[c]
(b) Activated calf thymus DNA	605	90
(c) $(rA)_n \cdot (dT)_{12-18}$	3960	50
(d) $(dA)_n \cdot (dT)_{12-18}$	100	5
(e) $(rC)_n \cdot (dG)_{12-18}$	445	120
(f) $(dC)_n \cdot (dG)_{12-18}$	3000	220
Ratio[d]		
a/b	0.04	0.39
c/d	39.60	10.00
e/f	0.15	0.55

[a] A 0.05-μg amount of purified SNV or CZAV DNA polymerases were incubated for 10 min at 37 C in the standard reaction mixture containing the optimum concentrations of divalent cation ($MgCl_2$ or $MnCl_2$) for each template·primer and each DNA polymerase.

[b] In the presence of actinomycin D (100 μg/ml).

[c] Optimal Mg^{2+} concentration (20 mM $MgCl_2$).

[d] Ratios were calculated from the rates listed in this table.

plate·primer relative to the rate with calf thymus DNA by CZAV DNA polymerase was 10 times greater than the relative rate by SNV DNA polymerase (Table 3, a and b). The rates of DNA synthesis with $(rA)_n \cdot (dT)_{12-18}$ template·primer were much greater than with $(dA)_n \cdot (dT)_{12-18}$ template·primer for both SNV DNA polymerase and CZAV DNA polymerase (Table 3, c and d). The rates of DNA synthesis by both DNA polymerases with $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ template·primers were high.

Hybridization of SNV [^{3}H]DNA to SNV RNA. SNV [^{3}H]DNAs were synthesized in the presence of actinomycin D (100 μg/ml) and either $MgCl_2$ or $MnCl_2$ by purified SNV or CZAV DNA polymerases with an SNV RNA (DNase I-treated total SNV RNA) template. All [^{3}H]DNAs were over 90% resistant to S1 nuclease digestion and became completely sensitive to S1 nuclease after alkali treatment. All [^{3}H]DNAs before denaturation sedimented with the template RNA in sucrose gradient centrifugations faster than 28*S*. After denaturation with alkali, [^{3}H]DNAs sedimented at 4*S* in sucrose gradient centrifugations. These [^{3}H]-DNAs were hybridized to SNV RNA as described above (Fig. 4). Less than 10% hybridi-

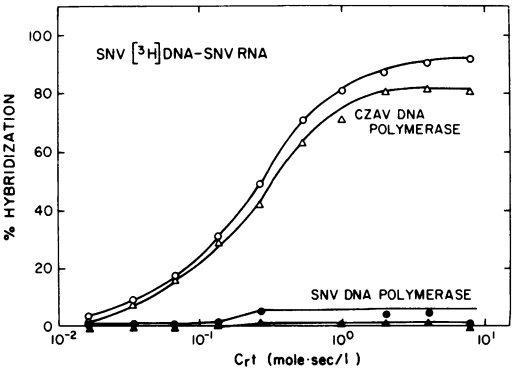

FIG. 4. *Hybridization of SNV [^{3}H]DNAs to SNV RNA. SNV [^{3}H]DNAs were prepared with purified SNV RNA and purified SNV DNA polymerase in the presence of $MgCl_2$ (●) or $MnCl_2$ (▲) and with purified SNV RNA and purified CZAV DNA polymerase in the presence of $MgCl_2$ (○) or $MnCl_2$ (△). Each SNV [^{3}H]DNA (2,000 to 3,000 dpm) was hybridized to SNV RNA as described in Materials and Methods. C,t values are corrected to a standard salt concentration (0.18 M NaCl) using values for DNA·DNA hybridization (1).*

zation was detected between SNV RNA and SNV [^{3}H]DNA synthesized by SNV DNA polymerase in the presence of $MgCl_2$ or $MnCl_2$, whereas SNV [^{3}H]DNA synthesized by CZAV DNA polymerase in the presence of $MgCl_2$ or $MnCl_2$ hybridized to SNV RNA under the same conditions over 90 and 80%, respectively. SNV [^{3}H]DNA synthesized by SNV DNA polymerase did not hybridize to poly(rA). SNV [^{3}H]DNA synthesized in an endogenous reaction in the presence of Mn^{2+} also hybridized to SNV RNA less than 20% under the same conditions of hybridization (data no shown). The lack of hybridization of SNV [^{3}H]DNA synthesized in vitro by SNV DNA polymerase may relate to a lack of fidelity of DNA synthesis by the SNV DNA polymerase (manuscript in preparation). However, the results of this experiment made it clear that there is a difference in ability to copy SNV RNA between SNV and CZAV DNA polymerases.

lymerase has RNase H activity apparently on the same molecule (4, 5). The purified SNV DNA polymerase was examined for RNase H activity. When [^{3}H]RNA·DNA prepared with *E. coli* RNA polymerase and heat-denatured calf thymus DNA was incubated with the DNA polymerases of SNV or CZAV, the [^{3}H]RNA strand was hydrolyzed (Fig. 5A). When the substrate was first heat denatured and then incubated with the DNA polymerases, the hydrolysis of the [^{3}H]RNA strand was much less (Fig. 5A). When [^{3}H]$(rA)_n \cdot (dT)_m$ was incubated with SNV or CZAV DNA polymerases, the [^{3}H]$(rA)_n$ strand of the hybrid was hydrolyzed, but the [^{3}H] $(rA)_n$ alone was not hydrolyzed by either DNA polymerase (Fig. 5B).

A divalent cation was required for RNase H activity, but the concentration was not critical. The optimal $MgCl_2$ concentration was about 1 to 2 mM (Fig. 6), but even at 10 mM $MgCl_2$ RNase H activity was still clearly detectable. $MnCl_2$ was more effective than $MgCl_2$, and the optimum concentration was even broader: 0.5 mM to 8 mM of $MnCl_2$ showed almost equal ac-

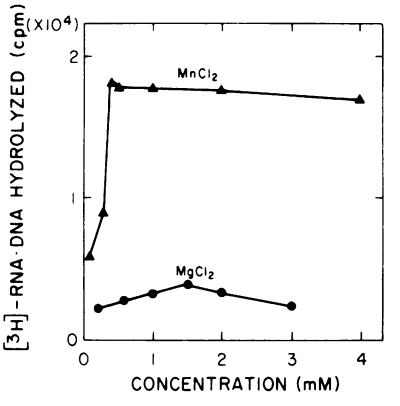

FIG. 6. *Optimum divalent cation concentrations for RNase H activity of SNV DNA polymerase. About 0.1 μg of SNV DNA polymerase was incubated at 37 C for 10 min with 8,000 dpm of [^{3}H]RNA-DNA as described in Materials and Methods at the indicated concentrations of $MgCl_2$ (●) or $MnCl_2$ (▲).*

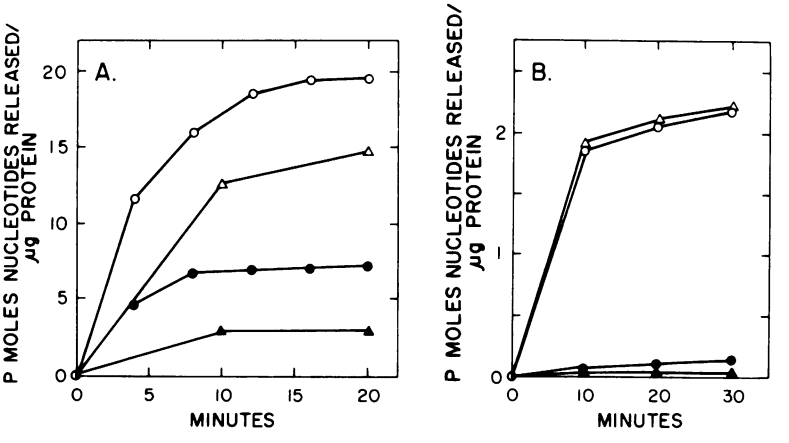

FIG. 5. *RNase H activity of SNV DNA polymerase. (A) A 0.2-μg amount of SNV DNA polymerase (○, ●) or CZAV DNA polymerase (△, ▲) was incubated with 7,500 dpm of [^{3}H]RNA·DNA (○, △) or heat-denatured [^{3}H]RNA·DNA (●, ▲) as described in Materials and Methods. Picomoles of nucleotide released per migrogram of protein were calculated for each time point. (B) A 2-μg amount of SNV DNA polymerase (○, ●) or CZAV DNA polymerase (△, ▲) was incubated with a mixture of 10,000 dpm of [^{3}H]$(rA)_n$ and $(dT)_m$ (○, △) or [^{3}H]$(rA)_n$ (●, ▲) as described in Materials and Methods. Picomoles of nucleotide (188 dpm) released per microgram of protein were calculated for each time point.*

tivities, but at higher than 10 mM the activity gradually decreased (Fig. 6, data not shown).

To test whether the RNase H and DNA polymerase activities were present on the same molecule, the purified SNV DNA polymerase was subjected to glycerol gradient centrifugation in the presence of 0.5 M KCl (Fig. 7). The DNA polymerase and RNase H activities were located in the same fractions in the gradient. This experiment indicated that SNV DNA polymerase, like AMV DNA polymerase, has RNase H and DNA polymerase activities on the same molecule.

The serological relationships of the RNase H activities of SNV DNA polymerase and of avian leukosis-sarcoma virus DNA polymerase were examined using antibodies prepared against AMV DNA polymerase (IgG[immunoglobulin G]-AMV) and SNV DNA polymerase (IgG-SNV) (Table 4). SNV DNA polymerase preincubated with control IgG or IgG-AMV digested

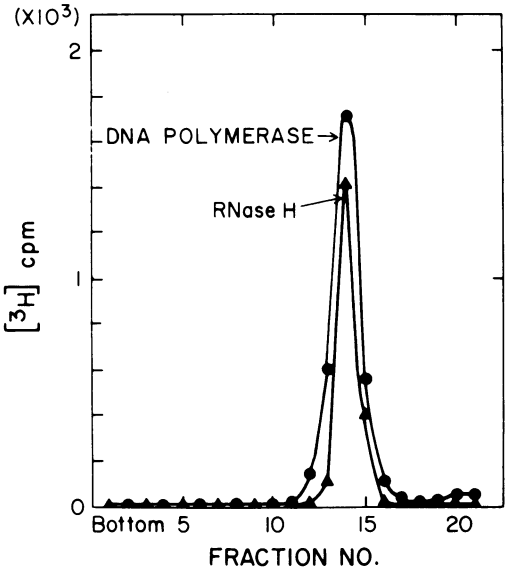

FIG. 7. *Glycerol gradient centrifugation of purified SNV DNA polymerase. A linear 15 to 45% (vol/vol) glycerol gradient (4.6 ml) in 0.02 M Tris-hydrochloride (pH 8.0) containing 0.001 M EDTA, 0.005 M dithiothreitol, and 0.5 M KCl was prepared in a tube of a Beckman SW50.1 rotor. SNV DNA polymerase (0.2 ml, ca. 2 μg) was placed on top of the gradient, and the gradient was centrifuged in a Spinco L centrifuge at 50,000 rpm for 15 h at 5 C. The gradient was fractionated from the bottom. The peak of DNA polymerase activity (●) was detected by a standard DNA polymerase assay containing* $(rA)_n \cdot (dT)_{12-18}$. *The peak of RNase H activity (▲) was detected by [³H]RNA·DNA digestion as described in Materials and Methods.*

TABLE 4. *Serological relationships of RNase H activities of SNV and CZAV DNA polymerases*[a]

DNA polymerase	Antibody	[³H]RNA·DNA hydrolyzed (dpm)	% Hydrolyzed
None	None	0	0
SNV	IgG-control	8740	97
	IgG-AMV	8630	96
	IgG-SNV	2500	28
CZAV	IgG-control	8540	95
	IgG-AMV	3110	34
	IgG-SNV	8480	94

[a] A 0.2-μg amount of SNV or CZAV DNA polymerase was preincubated in 25 μl at room temperature for 20 min with 50 μg of control γ-globulin (IgG-control), antibody against AMV DNA polymerase (IgG-AMV), or antibody against SNV DNA polymerase (IgG-SNV). The RNase H activity remaining was assayed with [³H]RNA·DNA as described in Materials and Methods. The reactions were carried out for 30 min. Trichloroacetic acid-soluble counts were calculated for each reaction by subtracting the trichloroacetic acid-insoluble counts from the trichloroacetic acid-insoluble counts (9,000 dpm) of the reaction without DNA polymerase and antibody.

about 95% of the [³H]RNA strand of [³H]RNA·DNA. However, when SNV DNA polymerase was preincubated with IgG-SNV, only 30% of the [³H]RNA strand of [³H]RNA·DNA was digested. When CZAV DNA polymerase was preincubated with IgG-AMV, only 35% of the [³H]RNA strand of [³H]RNA·DNA was digested, whereas 95% of the [³H]RNA strand of [³H]RNA·DNA was digested when the DNA polymerase was preincubated with control IgG or IgG SNV. Therefore, the RNase H activity of SNV DNA polymerase is serologically distinct from that of avian leukosis-sarcoma virus DNA polymerase as is the DNA polymerase activity (15).

DISCUSSION

The properties of the purified DNA polymerase of SNV are described in this paper. SNV is a ribodeoxyvirus and a member of the REV group. It has been proposed that both groups of avian ribodeoxyviruses, ALV and REV, originated from the avian cell genome (22), and small homologies remain between the two groups of viruses. However, if this hypothesis is true, REV seem to have diverged more than ALV from the avian cell genome. For example: REV RNAs have less nucleotide sequence homology to avian cell DNA than ALV RNAs (9), and REV spread horizontally in fowl and cause acute

infections in fowl and cytopathic effects in avian fibroblasts in cell culture (23).

The purified DNA polymerase activity of SNV is a single polypeptide with a molecular weight of 68,000. $MgCl_2$ is the preferred divalent cation for DNA synthesis on a deoxyribohomopolymer template, and $MnCl_2$ is the preferred divalent cation for DNA synthesis on an RNA template.

These preferences for Mg^{2+} and Mn^{2+} are similar to those of other ribodeoxyvirus DNA polymerases (6). The relative rates of DNA synthesis with different template·primers are similar to other ribodeoxyvirus DNA polymerases and only slightly different quantitatively from chicken cellular DNA polymerases (13).

[^{3}H]DNA synthesized by SNV DNA polymerase using an SNV RNA template did not hybridize to SNV RNA. In the same experiment, [^{3}H]DNA synthesized by CZAV DNA polymerase using an SNV RNA template almost completely hybridized to SNV RNA.

One possible explanation for this result is a lack of proper primer molecules associated with SNV RNA. Although the SNV RNA was a good template·primer for ALV DNA polymerases, SNV DNA polymerase may require different primer molecules. Consistent with this hypothesis was the poor stimulation of DNA synthesis when $(dT)_{12-18}$ was added to the reaction mixture and the presence of an RNA polymerase activity in SNV virions (15a).

Other possible explanations for these results are that the SNV DNA polymerase was altered in the course of purification or that it needs to be processed or another molecule added to it in the infected cells before efficient RNA-directed DNA synthesis occurs. Possibly related to these hypotheses is the observation that the ALV DNA polymerase has two subunits, and the smaller subunit may be derived by proteolytic cleavage from the larger one (3, 17). By contrast, however, some mammalian ribodeoxyvirus DNA polymerases have efficient RNA-directed DNA polymerase activity with a single polypeptide of about 70,000 daltons (6, 16). Consistent with these hypotheses is the low fidelity observed for DNA synthesis by SNV DNA polymerase with single-stranded homopolymer templates (S. Mizutani, and H. M. Temin, manuscript in preparation).

Like ALV DNA polymerases, REV DNA polymerases apparently have RNase H activity on the DNA polymerase molecule. Moelling et al. also have observed RNase H activity in the partially purified DNA polymerase of reticuloendotheliosis virus (strain T) (18). Antibody against purified SNV DNA polymerase neutralized the RNase H activity of SNV DNA polymerase, but antibody against AMV (an ALV) DNA polymerase did not neutralize this activity. The reciprocal was also true. Therefore, the RNase H activity has group-specific antigenic determinants.

The function of the RNase H activity of ribodeoxyvirus DNA polymerases is not fully understood. It has been suggested that the RNase H activity of the ribodeoxyvirus DNA polymerase has a function in synthesis of double-stranded DNA by digesting the RNA strand of an RNA-DNA hybrid (12).

A class of DNA polymerases with RNase H activity on the same molecules has now been described in two different groups of avian ribodeoxyviruses, ALV and reticuloendotheliosis viruses. Both groups may have evolved from avian cells (22). Therefore, this same class of DNA polymerases may exist in uninfected cells and have a function different than that of other cellular DNA polymerases. Alternatively, the REV DNA polymerases could have resulted from fusion of cellular genes for DNA polymerase and for RNase H.

The results in this paper may indicate that the presence of RNase H activity is a better test for a DNA polymerase involved in RNA-directed DNA synthesis than rates of synthesis with different template·primers. The failure to copy in a test tube heteropolymer RNA into DNA that hybridizes to the same RNA indicates that presently unknown events are involved in RNA-directed DNA polymerase activity in vivo.

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