terpretation and the reasonable agreement with the data. In either case, studies with iodoglucagon provide good evidence for the following: (i) the tyrosyl residues play a crucial role in the binding and action of glucagon, in accord with theoretical considerations (Chou and Fasman, 1975) and the x-ray crystallographic analysis (Sasaki et al., 1975); (ii) ionization of the phenoxy groups appears to have important deleterious effects on the structure required for the binding and action of the hormone, possibly through loss of hydrogen binding; and (iii) incorporation of an iodine atom modifies the nature of glucagon binding and action to give a more potent analogue.

Acknowledgments

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HeLa Cell DNA Polymerase γ: Further Purification and Properties of the Enzyme†

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ABSTRACT: DNA polymerase γ has been purified over 60 000-fold from HeLa cells which contain no detectable type C viral particles. This purified enzyme shows a specific activity of 25 000 units/mg of protein which is comparable to the known specific activity of homogeneous preparations of human α and β polymerases. The isolated enzyme shows apparent molecular weights ranging from 160 000 to 330 000 according to the method of analysis. The enzyme exhibits optimal activity for copying poly(A) in the presence of 50 mM KPO_4 and 130 mM KCl and, under these conditions, copies poly(A) 20 times more rapidly than activated DNA. These assay conditions permit a clear distinction between the γ-polymerase and DNA polymerase β which is markedly inhibited by phosphate at this concentration. A comparison of the copying of activated DNA, poly(dA) and poly(A) by DNA polymerases α, β, and γ under optimal assay conditions for each enzyme is presented. Studies with synthetic and natural nucleic acid templates also show the γ-polymerase to behave differently than the reverse transcriptases of avian myeloblastosis virus or Rauscher leukemia virus.

There are now four presumptive DNA polymerases in mammalian cells (Weissbach, 1975). Three of the enzymes, DNA polymerases α, β, γ, are presumed to be involved in the replication or repair of nuclear DNA, whereas the mitochondrial DNA polymerase is associated with the organelle and is assumed to participate in mitochondrial DNA synthesis. The γ-polymerase is relatively efficient in its use of synthetic RNA templates such as poly(A), a property not unlike that shown by the reverse transcriptases of the type C tumor viruses. Thus, one of the objectives of this study was to obtain highly purified preparations of DNA polymerase γ in order to compare the cellular enzyme to the viral enzyme.

We have now purified human DNA polymerase γ more than 60 000-fold from HeLa cells which are free of demonstrable oncorna virus particles. The optimal conditions needed for the copying of various synthetic and natural DNA and synthetic RNA templates by this enzyme preparation have been studied. These studies have provided a clearer picture of the differences between DNA polymerases α, β, and γ, and the reverse transcriptase of oncorna viruses, but have not enabled us to detect the copying of natural RNA by the HeLa cell γ-polymerase.

Materials and Methods

[^{3}H]Deoxynucleoside triphosphates were purchased from Schwarz/Mann, Orangeburg, N.Y. The oligonucleotides $(dA)_{12-18}$, $(dC)_{12-18}$, and $(dG)_{12-18}$ were obtained from Collaborative Research, Inc., Waltham, Mass.; $(dT)_{12-18}$ was supplied by Schwarz/Mann. $(A)_{12-18}$ was prepared from reovirus RNA acccrding to Stoltzfus and Banerjee (1972). Poly(A), poly(dA), poly(C), poly(dC), poly(I), poly(dT), and

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poly(U) were obtained from Miles Laboratories, Kankakee, Ill. Activated salmon sperm DNA was prepared as previously described (Fry and Weissbach, 1973). Oligonucleotides from HeLa DNA were prepared according to Richardson and Kornberg (1964). HeLa DNA was prepared by the method of Poonian et al. (1971). HeLa nuclear RNA was prepared by lysis of the nuclei with Pronase and sodium dodecyl sulfate (LaColla and Weissbach, 1975). The lysed nuclei were extracted twice with phenol–chloroform (1:1) and the aqueous phase was precipitated with 2 volumes of ethanol. The nucleic acid precipitate was redissolved in 0.02 M Tris,[1] pH 7.6, 0.005 M $MgCl_2$ and treated with DNase I (20 μg/ml). The treatment with phenol–chloroform, ethanol precipitation, and DNase I digestion was repeated once again and the final nuclear RNA preparation was stored in 0.02 M Tris, pH 7.6, at −20 °C. HeLa RNA, containing poly(A), was prepared following the method of Banerjee and Rhodes (1973) using total HeLa cell RNA isolated by the method of Glišin et al. (1974) as the starting material. Bovine serum albumin was purchased from Biotest-Serum Institut GmbH, Frankfurt, Germany. Calf thymus histone f_1 was a gift from Dr. M. Modak of the Sloan Kettering Institute.

Diethylaminoethylcellulose (DE-52) and phosphocellulose (P11) were supplied by Whatman; hydroxylapatite Bio-Gel HT was obtained from Bio-Rad; Ultrogel AcA22 was a gift of LKB. Native DNA-cellulose was prepared by the method of Litman (1968) using native calf thymus DNA and cellulose powder No. 410 (Grycksbo Pappersbruk AB, Sweden). The cellulose powder was washed before use with 0.5 M KPO_4, pH 7.5, followed by a wash with 0.02 M KPO_4, pH 7.5.

Avian myeloblastosis virus (AMV) was obtained from Dr. J. Beard, Duke University, Durham, N.C., and was further purified as described by Kacian et al. (1971). Highly purified AMV DNA polymerase was a gift of Dr. L. A. Loeb (Institute for Cancer Research, Fox Chase, Philadelphia, Pa.).

Rauscher leukemia virus (RLV) BALB/c mouse plasma: citrate was from University Laboratories, Inc., Highland Park, N.J., and was the kind gift of Dr. D. Kacian, Columbia University.

The following protein and enzyme markers were used in acrylamide gel electrophoresis and velocity sedimentation studies: bovine serum albumin (Miles Laboratories); beef liver catalase (Worthington Biochemical Corp.); and lactate dehydrogenase (LDH) (pig heart; Boehringer Mannheim GmbH, Germany).

Growth of Cells. HeLa S-3 cells were grown in suspension and harvested as previously described (Spadari and Weissbach, 1974).

Purification of DNA Polymerases γ. Unless otherwise noted, all purification procedures were carried out at 0–4 °C. All buffers contained 0.5 mM dithiothreitol, and starting with the phosphocellulose chromatography they contained, in addition, 20% (v/v) glycerol and 0.2% Nonidet (N-P40). The dialysis buffer used was 0.02 M potassium phosphate (pH 7.5) containing 20% (v/v) glycerol, 0.4% N-P40, 0.2% Triton N-101, and 1 mM dithiothreitol.

Preparation of Cell Fractions. Eighty grams of frozen HeLa S-3 cells was suspended in 300 ml of 10 mM NaCl, 1 mM potassium phosphate (pH 7.8), and after 30 min at 0 °C the cells were broken with a Dounce homogenizer. The nuclei were pelleted from the cytoplasm by centrifugation (1000*g*, 10 min). The nuclear pellet was further homogenized with 500 ml of a solution containing 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate (pH 6.8), and 0.3% Triton N-101, and centrifuged for 10 min at 1000*g*. A second Triton wash of the nuclear pellet was carried out under the same conditions and both supernatants were combined with the original cytoplasmic fraction.

DEAE-Cellulose Chromatography. The combined cytoplasmic fractions (1800 ml) were loaded directly onto a 800-ml column of DEAE-cellulose which was equilibrated with 0.02 M potassium phosphate (pH 7.5). After a wash with 1.25 column volumes of the same buffer, the column was eluted in 20-ml fractions with 2.5 column volumes of a linear gradient from 0.02 to 0.3 M potassium phosphate (pH 7.5). Using $(dT)_{12-18}$•poly(A) as a template, DNA polymerase γ activity was detected as a single peak at 0.15 M potassium phosphate.

Phosphocellulose Chromatography. The peak fractions (500 ml) of DNA polymerase γ obtained from DEAE-cellulose chromatography were pooled. Then phosphocellulose (150 ml), equilibrated in 0.01 M potassium phosphate (pH 7.5), was added with stirring, and the mixture was then further diluted by addition of 1 l. of 0.01 M potassium phosphate (pH 7.5). After 4 h of stirring the slurry was placed in a column, and the resin was washed with 2 column volumes of dilution buffer. The column was eluted in 7.5-ml fractions with 4 column volumes of a linear gradient from 0.1 to 0.4 M potassium phosphate (pH 7.5). DNA polymerase γ activity appeared as a single peak at 0.26 M potassium phosphate.

Hydroxylapatite Chromatography. The peak fractions (158 ml) of DNA polymerase γ from the P-11 chromatography were dialyzed and then applied onto a hydroxylapatite column (20 ml) which was equilibrated with 0.02 M potassium phosphate (pH 7.5). After a wash with 2 column volumes of the same buffer, the column was eluted in 1.85-ml fractions with 5 column volumes of a linear gradient from 0.05 to 0.4 M potassium phosphate (pH 7.5). DNA polymerase γ activity appeared at 0.175 M potassium phosphate.

AcA 22-Hydroxylapatite Chromatography. The peak fractions (16 ml) from hydroxylapatite chromatography were dialyzed and applied to a column consisting of an Ultrogel AcA 22 resin (11.5 × 2.4 cm) with a top layer of hydroxylapatite (1 cm height). The column was washed with 1 column volume of the dialyzing buffer, without Triton N-101, and the adsorbed protein was eluted in 1.5-ml fractions with 4 column volumes of a linear gradient from 0.02 to 0.4 M potassium phosphate containing 0.2% N-P40. DNA polymerase γ was eluted at 0.16 M potassium phosphate.

DNA-Cellulose Chromatography. After dialysis, the active fractions of DNA polymerase γ from AcA 22-hydroxylapatite chromatography (15 ml total) were applied to a 4-ml DNA-cellulose column previously equilibrated with 0.02 M potassium phosphate (pH 7.5) containing 1 mM dithiothreitol. After a wash with 10 column volumes, the column was eluted in 0.85-ml fractions with 25 column volumes of a linear gradient from 0.02 to 0.4 M potassium phosphate (pH 7.5) containing 1 mM dithiothreitol. DNA polymerase γ activity was detected at 0.32 M potassium phosphate.

Concentration of the Enzyme. To avoid any dialysis step, which led to loss of enzyme activity, the peak fractions of DNA polymerase γ from the DNA-cellulose chromatography were diluted 1 to 7 with 10 mM potassium phosphate (pH 7.5) and applied to a 1-ml hydroxylapatite column. The enzyme was eluted either in one step with 0.4 M potassium phosphate (pH

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; LDH, lactate dehydrogenase; P_i, inorganic phosphate; NEM, *N*-ethylmaleimide; DEAE, diethylaminoethyl.

7.5) containing 40% (v/v) glycerol and 1 mM dithiothreitol or with a linear gradient of 20 column volumes from 0.02 to 0.4 M potassium phosphate.

Assay for DNA Polymerase γ. The reaction mixture contained, in a final volume of 100 μl, 50 μg of bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 100 mM KCl, 2.5 mM dithiothreitol, 5 μg of $(dT)_{12-18}$·poly(A), and 0.1 mM [^{3}H]dTTP with a specific activity of 100 cpm/pmol. The incubation was carried out at 30 °C for 15 min. When assays contained activated salmon sperm DNA as a template, incubation was carried out at 37 °C for 15 min and the reaction mixture contained in a final volume of 100 μl, 50 μg of bovine serum albumin, 50 mM Tris-HCl (pH 8.5), 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 μg of activated salmon sperm DNA, 0.1 mM dATP, dCTP, dGTP, and [^{3}H]dTTP with a specific activity of 100 cpm/pmol. The radioactivity incorporated into an acid-insoluble form was assayed as previously described (Berns et al., 1969). One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol of TTP into a polymeric form in 60 min at 30 °C with a $(dT)_{12-18}$·poly(A) template.

Assay for Pyrophosphate Exchange Activity of DNA Polymerase γ. The PP_i-exchange activity under optimal assay conditions was determined according to Weiss et al. (1968) in the presence of 0.1 mM $^{32}PP_i$ (specific activity, 65 cpm/pmol).

Purification and Assay of DNA Polymerase α *and* β. The purification and the assay of DNA polymerases α and β were carried out as previously described (Spadari and Weissbach, 1974). One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmol of substrate in 60 min at 37 °C with 250 μg/ml activated salmon sperm DNA. The specific activity of a routine DNA polymerase α preparation ranged between 5000 and 10 000 units/mg; the specific activity of polymerase β was about 1000 to 2000 units/mg. The enzymes were characterized in respect to *N*-ethylmaleimide (NEM) sensitivity, salt inhibition, reaction with anti-α-polymerase serum, and size in high salt glycerol gradient sedimentation runs.

Composition of Synthetic Templates. Oligonucleotides were annealed to homopolymers at a proportion (by weight) of three parts of oligonucleotide to seven parts of homopolymer (Fridlender and Weissbach, 1971).

Protein Determination. Protein was determined by the method of Böhlen et al. (1973) with bovine albumin as a standard.

Gel Electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was carried out at pH 8.9 as described by Davis (1964) but without the stacking gel. The sample buffer contained 50 mM potassium phosphate, 10 mM 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue and 2–5 μg of enzyme. To remove any possible DNA contamination, the enzymes were treated with DNase I before electrophoresis. For DNase treatment of the enzyme, the sample buffer contained in addition 5 mM $MgCl_2$ and 25 μg/ml pancreatic DNase. After 3-min incubation at 37 °C, the samples were chilled in ice and immediately loaded on the gels and covered with reservoir buffer containing 10 mM 2-mercaptoethanol. Electrophoresis was performed at 1 mA/gel for 1 h and then at 2.5 mA/gel until the marker dye reached the bottom of the gel. In each gel run, marker proteins were electrophoresed in parallel gels. For determination of DNA polymerase activity, the gels were crushed in 2-mm portions with a Gilson gel fractionator. Each gel fraction was mixed with 0.4–0.5 ml of 0.2 M potassium phosphate (pH 7.5) containing 1 mM dithiothreitol, 20% (v/v) glycerol, and 0.1% N-P40, and incubated overnight by shaking at 4 °C. The recovery of enzyme activity varied between 80 and 90%. The molecular weight of the DNA polymerases was determined according to Hedrick and Smith (1968) by comparison of their mobilities using 4, 5, and 6% acrylamide gels in relationship to the mobility of the marker proteins.

Results

Purification of DNA Polymerase γ. DNA polymerase γ has been previously purified about 3500-fold from HeLa S-3 cells using DEAE-cellulose, phosphocellulose, and hydroxylapatite as the chromatographic steps (Spadari and Weissbach, 1974). The enzyme at this stage was unable to copy natural RNA molecules which had been shown to be effective templates for the reverse transcriptases of the RNA tumor viruses. To fully examine the template characteristics of DNA polymerase γ, we decided to further purify the enzyme as described below. The purification steps up to the hydroxylapatite chromatography were essentially the same as already described (Spadari and Weissbach, 1974), except that a batch technique was applied (see Materials and Methods). It should be mentioned that DNA polymerase γ appeared as a single homogeneous peak eluting from DEAE-cellulose and phosphocellulose column by this technique. Splitting of the enzyme activity into two peaks as previously reported (Spadari and Weissbach, 1974) was not observed under these conditions. Figure 1 shows the additional purification steps, starting with the hydroxylapatite chromatography step, which were undertaken. Panels A to C illustrate respectively hydroxylapatite, the AcA 22–hydroxylapatite, and DNA-cellulose chromatograms of DNA polymerase γ obtained from the cytoplasmic fraction of the cell. In the hydroxylapatite chromatography, the bulk of the enzyme activity utilizing activated DNA as a template was found to elute at 0.13 M, whereas the γ-polymerase activity copying $(dT)_{12-18}$·poly(A) was found to elute at 0.18 M potassium phosphate (panel A). The peak of enzyme activity which copied activated DNA clearly showed a different elution pattern from that which copied $(dT)_{12-18}$·poly(A). We assumed this to be due to the presence of DNA polymerase α in our γ-polymerase preparation. Therefore, the peak fractions of enzymatic activity which copied $(dT)_{12-18}$·poly(A) were combined and chromatographed on a column of Ultrogel AcA 22–hydroxylapatite (Figure 1B), which provides both gel filtration and adsorption conditions. This step also resulted in a partial separation of the two enzymatic activities which read activated DNA and $(dT)_{12-18}$·poly(A), respectively. It should be noted that attempts to further separate the two activities by rechromatography on a second hydroxylapatite column or a AcA 22 column with a DEAE-cellulose top layer failed. However, it was found that the affinity chromatography on native DNA-cellulose (Figure 1C) provided an effective separation of the γ-polymerase from the other enzymatic activity which copied only activated DNA and led to a considerable purification (Table I). In this step, the enzyme which copied $(dT)_{12-18}$·poly(A) eluted at approximately 0.32 M KPO_4 (panel C), whereas the activity reading activated DNA eluted at 0.15 M KPO_4 and 0.32 M KPO_4. The γ-polymerase activity, eluting at 0.32 M KPO_4, copied the poly(A) template (50 μg/ml) at about five times the rate it showed with activated DNA (250 μg/ml). Table I summarizes the purification of the cytoplasmic DNA polymerase γ. This enzyme appears to be identical with DNA polymerase γ peak II previously reported by Spadari and Weissbach (1974). At the DNA-cellulose step, the enzyme showed a specific activity of about 25 000 units/mg

TABLE I: Purification of HeLa Cell DNA Polymerase γ.[a]

Fraction	Total Protein (mg)	Total Act. (Units)	Spec Act. Units/mg Protein)	Purification
Cytoplasm	7100.0	2924	0.41	1
DEAE-Cellulose	682.2	9138	13.3	32
Phosphocellulose	167.0	6964	41.7	102
Hydroxylapatite	9.6	6342	660.6	1611
AcA 22-hydroxylapatite	2.3	2328	1012.2	2469
DNA-Cellulose	0.045	1130	25111.1	61247

[a] DNA polymerase γ activity was assayed as described in Materials and Methods.

protein and was purified about 60 000-fold. In our work, the enzyme was further concentrated by stepwise elution from the hydroxylapatite column with 0.4 M potassium phosphate containing 40% glycerol, 1 mM dithiothreitol, and 0.1% N-P40 as described in Materials and Methods. Stored under these conditions, the enzyme was stable for several months at −20 °C. In the last purification step it was advantageous to work with siliconized glassware and plastic tubes to avoid adsorption of the enzyme on glass. All of the following studies concerning the properties of the enzyme were carried out with enzyme preparations concentrated by hydroxylapatite.

Size of DNA Polymerase γ under Native Conditions. Further purification of the enzyme was undertaken using a preparative gel electrophoresis under nondenaturing conditions. It was found that under our conditions HeLa DNA polymerases α and γ entered the gels which were run at pH 8.9. DNA polymerase β, known to possess a p*I* of about 9.3 (Wang et al., 1975), does not enter the gels under these conditions. Figure 2 demonstrates the result of the preparative gel electrophoresis. The DNA polymerase γ activity (panel A) migrated with a relative mobility which was slightly slower than that of catalase. Staining of parallel gels containing the purified γ-polymerase preparation showed that about 10% of the stained protein corresponded to the activity peak, and most of the stained protein was found on the top of the gels (fractions 1 to 4 of the gel presented in Figure 2, panel A). In a more extended gel run (panel B) DNA polymerase γ showed a main peak and a shoulder with either $(dT)_{12-18}$·poly(A) or activated DNA as a template. In this experiment, DNA polymerase α, isolated from nuclei, appeared as a single peak of activity migrating at a faster relative mobility than the catalase marker (Figure 2B).

We have also examined the most purified enzyme preparation obtained from the nondenaturing gel electrophoresis (Figure 2A, fraction 11) in sodium dodecyl sulfate-acrylamide gels. Two major protein bands of 65 000 and 56 000 daltons and two minor bands at 25 000 and 45 000 daltons were observed. However, we have been unable to identify which of these peptides is derived from DNA polymerase γ.

To get an approximate idea of the size of the DNA polymerases, molecular weight determinations under nondenaturing conditions were carried out as described in Materials and Methods using 4, 5, and 6% gels (Hedrick and Smith, 1968). DNA polymerase γ was found to show one peak with an approximate molecular weight of 274 000 ± 8000, whereas the molecular weight of DNA polymerase α was determined to be between 164 000 and 182 000. However, in gel filtration studies with Sephadex G-200, DNA polymerase γ was found

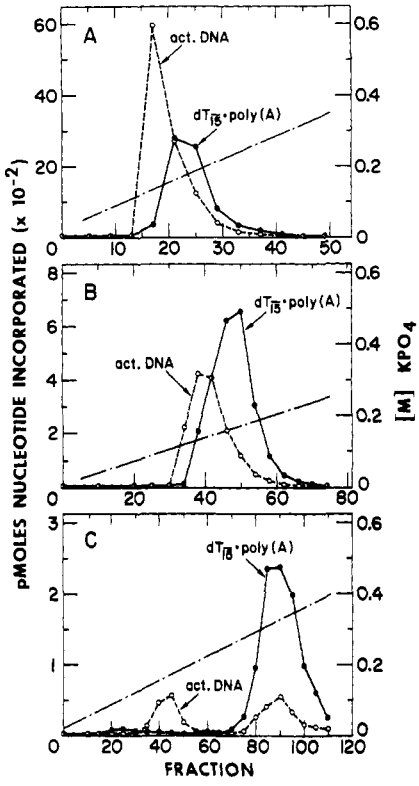

FIGURE 1: Column chromatography of HeLa cell DNA polymerase γ. (A) Hydroxylapatite chromatogram of DNA polymerase γ after the phosphocellulose chromatography step. An aliquot of the DNA polymerase peak (158 ml, fractions 40–60) from the phosphocellulose chromatography was subjected to a hydroxylapatite chromatography as described under Materials and Methods. Twenty-five-microliter samples of the indicated fractions were assayed for DNA polymerase activity with $(dT)_{12-18}$·poly(A) (●—●) and activated salmon sperm DNA (○- - -○) as templates as described in Materials and Methods. (B) Ultrogel AcA-22-hydroxylapatite chromatography. An aliquot of the DNA polymerase peak (panel A, fractions 21–33), containing 9.6 mg of protein, was chromatographed on a 56-ml Ultrogel AcA-22-hydroxylapatite column and 20-μl samples of the indicated fractions were assayed for DNA polymerase activity as described in part A. (C) DNA-cellulose chromatography. An aliquot of the DNA polymerase peak from panel B (fractions 45–55), representing 1.7 mg of protein, was applied to a 4-ml double-stranded DNA-cellulose column. After elution, 20-μl samples of the indicated fractions (0.85 ml) were assayed as described in part A.

to exhibit an apparent molecular weight of 330 000 ± 10 000 in either low salt (0.05 M potassium phosphate) or high salt (0.4 M potassium phosphate) conditions. Sedimentation analysis of the enzyme carried out in high salt containing glycerol density gradients [10–30% (v/v) glycerol in 0.25 M KPO_4 (pH 8.0), 0.5 mM dithiothreitol, 0.5 mM EDTA] revealed a sedimentation coefficient of 7.8 ± 0.2 S when catalase (11.5 S), lactate dehydrogenase (6.93 S), and bovine serum albumin (4.4 S) were used as the protein standards. From this sedimentation coefficient a molecular weight of 160 000 ± 5000 was calculated assuming a globular shape of the protein with a partial specific volume of 0.736 (Martin and Ames, 1961). The discrepancy in the molecular weights as determined by velocity sedimentation vis-à-vis acrylamide gel electrophoresis or gel exclusion techniques may indicate the γ-polymerase can exist in highly asymmetric or aggregated forms.

Properties of Purified DNA Polymerase γ

Effect of Salt. All assays were performed at a substrate concentration of 0.1 mM per deoxynucleoside triphosphate and 50 μg/ml of $(dT)_{12-18}$·poly(A). Figure 3 demonstrates the effect of salt on the DNA polymerase γ activity using

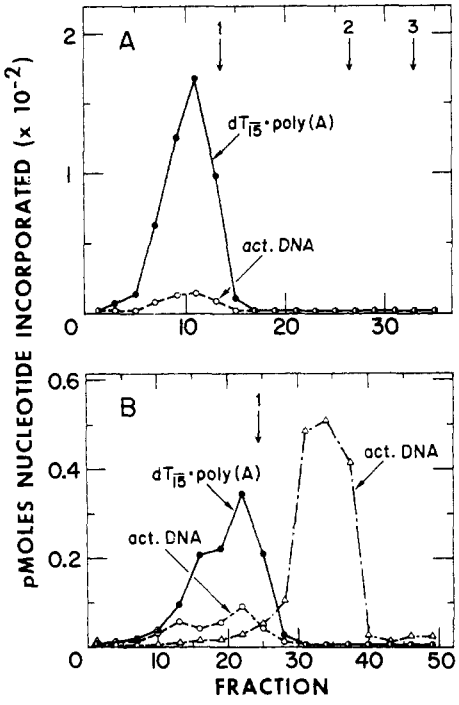

FIGURE 2: Polyacrylamide gel electrophoresis of DNA polymerase α and γ under nondenaturing conditions. The conditions for polyacrylamide gel electrophoresis were as described in Materials and Methods. The enzymes were pretreated with DNase I before electrophoresis. In the experiment shown in panel A, electrophoresis was performed at 1 mA per gel for 1 h and 2.5 mA per gel for 2.5 h, and in panel B electrophoresis was at 2.5 mA per gel for about 8 h. Migration of proteins is from left to right as shown. Panel A shows the electrophoresis of 2 μg of DNA polymerase γ in a 5% acrylamide gel. After the protein elution step, the DNA polymerase activity in 25-μl aliquots of the indicated fractions (0.5 ml) was assayed with $(dT)_{12-18}$·poly(A) (●—●) and activated salmon sperm DNA (○- - -○) as templates as described in legend of Table II. The internal and external protein standards used were (1) catalase (244 000 daltons), (2) LDH (136 000 daltons), and (3) bovine serum albumin (68 000 daltons). Panel B shows the electrophoresis of DNA polymerase α (5 μg) and γ (2 μg) under extended conditions. Both DNA polymerases were electrophoresed in separate gels with catalase (1) as an internal marker. Catalase activity was assayed as already described (Beers and Sizer, 1952). Twenty-five-microliter aliquots of the indicated fractions were assayed for DNA polymerase γ activity with $(dT)_{12-18}$·poly(A) (●—●) and activated DNA (○- - -○) as described in panel A. DNA polymerase α activity (△—△) in 15-μl aliquots was determined as described in Materials and Methods using activated DNA as a template.

$(dT)_{12-18}$·poly(A) as a template. In this experiment a combination of different KCl and potassium phosphate concentrations was used. The enzyme activity was determined to be the highest in the presence of 50 mM potassium phosphate and 100 to 150 mM KCl. We used 130 mM KCl as optimal concentration in our assays.

pH Optimum. The optimal pH for the DNA polymerase γ activity with a $(dT)_{12-18}$·poly(A) template was determined to be 8.5 under the above-mentioned high salt conditions.

Effect of Temperature. With optimal pH and salt conditions the DNA polymerase γ activity showed a relatively sharp peak at 42 °C. An Arrhenius plot of the temperature data revealed a linear relationship between 10 to 42 °C and an activation energy of about 15 kcal per mol. Our assays were carried out at 37 °C even though the activity at this temperature was 30% less than that seen at 42 °C.

Effect of Divalent Cations. The DNA polymerase activity with $(dT)_{12-18}$·poly(A) and activated DNA templates is dependent upon the presence of a divalent cation in the reaction mixture. The DNA polymerase activity with $(dT)_{12-18}$·poly(A)

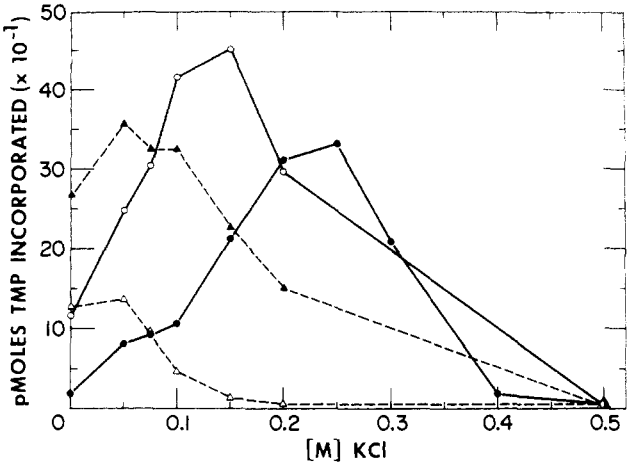

FIGURE 3: Effect of salt on DNA polymerase γ activity. The reaction mixtures were as described under Materials and Methods, except that various combinations of potassium phosphate and KCl concentrations were used as indicated: (●—●) 10 mM; (○—○) 50 mM; (▲- - -▲) 100 mM; and (△- - -△) 200 mM potassium phosphate.

shows an optimal Mn^{2+} concentration of 0.5 mM, and the activity with activated DNA exhibits an optimal Mg^{2+} concentration of 5 mM. Higher concentrations of divalent cations were found to be inhibitory.

Effects of Inhibitors and Further Properties. The DNA polymerase activity with $(dT)_{12-18}$·poly(A) and activated DNA was inhibited to about 40–50% by 1 mM *N*-ethylmaleimide and completely by 1 mM *p*-hydroxychloromercuribenzoate when the dithiothreitol concentration in the assay was reduced to 0.05 mM. The enzyme activity with $(dT)_{12-18}$·poly(A) was inhibited down to about 5% of its original activity by 1 mM sodium pyrophosphate whereas the copying of activated DNA was only depressed to 25% of the original under these conditions. These divergent inhibition values may only reflect the different concentrations of divalent cations used in these assays since raising the Mn^{2+} concentration about ten-fold resulted in an inhibition to about 30% with the $(dT)_{12-18}$·poly(A) template. Phosphonoacetic acid inhibited the copying of both templates to the same extent, i.e., 25% at 1 mM and 70–80% at 5 mM concentration. DNA polymerase γ did not catalyze any significant amount of PP_i exchange when the PP_i-exchange reaction was carried out in the presence of 0.1 mM $^{32}PP_i$ (specific activity 65 cpm/pmol) with or without $(dT)_{12-18}$·poly(A) and activated DNA under optimal assay conditions. Dr. Lawrence Loeb of the Institute for Cancer Research, Fox Chase, Philadelphia, Pa., has also tested the HeLa cell γ-polymerase for pyrophosphate exchange, using poly[d(A-T)] and $(dT)_{12-18}$·poly(A) templates with similar results.

Effect of Protease. To determine if the active site for $(dT)_{12-18}$·poly(A) and activated DNA activity were identical, we tried to selectively effect one or the other of the activities with the proteases α-chymotrypsin and trypsin. Two-tenths of a microgram of DNA polymerase γ was preincubated with 10 μg/ml of each protease for 15 min. At selected times, aliquots were removed and assayed on both templates as described under Materials and Methods. We found that the DNA polymerase activity with both templates dropped at the same rate. After 2-min preincubation, 50–60% of the initial activity was lost and total activity was lost after 15 min of protease treatment.

Template Specificity. The ability of DNA polymerase γ to utilize various synthetic and natural templates in comparison

TABLE II: Template Studies with HeLa Cell γ-Polymerase and AMV and RLV Reverse Transcriptases.[a]

Template	^{3}H-Labeled Substrate	γ-Polymerase Mn^{2+} (%)	γ-Polymerase Mg^{2+} (%)	AMV Purified Polymerase (%)	AMV Virion (%)	RLV Virion (%)
None		0.0	0.0	0.0	1.47	0.56
$(dT)_{12-18}$·poly(A)	dTTP	100.0	100.0	100.0	100.0	100.0
$(dG)_{12-18}$·poly(C)	dGTP	0.1	0.1	104.7	171.0	26.7
$(dC)_{12-18}$·poly(I)	dCTP	6.8	13.2	37.9	194.3	23.0
$(dA)_{12-18}$·poly(U)	dATP	0.2	0.2	0.1	0.1	0.2
$(rA)_{12-18}$·poly(dT)	dATP	13.9	24.2	0.05	0.1	0.1
$(dT)_{12-18}$·poly(dA)	dTTP	17.4	55.7	0.3	0.7	0.1
$(dG)_{12-18}$·poly(dC)	dGTP	23.3	65.3	34.5	98.8	132.7
Activated salmon sperm DNA	dTTP					
50 μg/ml		4.9	19.4	25.6	101.1	17.1
250 μg/ml		9.5	33.9	38.6	151.4	31.6
HeLa DNA-oligodeoxynucleotides	dTTP	4.5	10.8	4.3	47.4	0.33
HeLa denatured DNA	dTTP	0.7	0.4	0.8	3.6	0.02
HeLa nuclear RNA-oligodeoxynucleotides	dTTP	0.0	0.0	2.1	7.6	1.7
HeLa poly(A) RNA·dT_{12-18}	dATP	0.0	0.0	15.7	3.3	1.1

[a] DNA polymerase γ assays were carried out under the following reaction conditions. The reaction mixture contained, in a final volume of 100 μl, 50 μg of bovine serum albumin, 50 mM Tris-HCl (pH 8.5), 50 mM potassium phosphate (pH 8.5), either 0.5 mM $MnCl_2$ or 5 mM $MgCl_2$, 130 mM KCl, 2.5 mM dithiothreitol, 5 μg of template unless otherwise noted, and 0.1 mM of the indicated tritium labeled substrate (specific activity 100 cpm/pmol). Reaction mixtures containing natural DNA or RNA templates were assayed in the presence of 0.1 mM of all four deoxyribonucleoside triphosphates. Reverse transcriptase assays were performed in the absence of phosphate under the above given reaction conditions except that 100 mM KCl and 5 mM dithiothreitol were used. Incubations were carried out at 37 °C for 30 min. The viral extracts used to assay for DNA polymerase activity were prepared according to Fridlender and Weissbach (1971). The quantity of protein in the assay and the 100% values are: DNA polymerase γ, 0.5 μg, 2090 pmol (Mn^{2+} conditions), and 1056 pmol (Mg^{2+} conditions); purified AMV DNA polymerase, 0.1 μg, 946 pmol; avian myeloblastosis virion, 8.8 μg, 730 pmol; Rauscher leukemia virion protein, 4.4 μg, 772 pmol.

with the reverse transcriptases from AMV and RLV is summarized in Table II. The table represents data which were obtained with one given template concentration, though in certain cases, different template concentrations were examined. DNA polymerase γ utilized $(dT)_{12-18}$·poly(A) under Mn^{2+} conditions about 20-fold better and under Mg^{2+} conditions about 5-fold better than activated salmon sperm DNA. The most effective template for the highly purified AMV DNA polymerase seems to be $(dG)_{12-18}$·poly(C) whereas the DNA polymerase activity found in the detergent-treated AMV virions prefers $(dC)_{12-18}$·poly(I) and the enzyme found in RLV virions prefers $(dG)_{12-18}$·poly(dC). No reading was achieved with $(dA)_{12-18}$·poly(U) with any of the DNA polymerases. Under these high salt conditions in the assay, $(dG)_{12-18}$·poly(C) was not copied by DNA polymerase γ. However, DNA polymerase γ is able to copy $(rA)_{12-18}$·poly(dT) and $(dT)_{12-18}$·poly(dA) very well, though these templates were not utilized by the reverse transcriptases. The templates $(dG)_{12-18}$·poly(dC) and $(dC)_{12-18}$·poly(I) were utilized quite well by all of the enzymes. Studies with highly purified RLV reverse transcriptase carried out by Modak and Marcus (personal communication) have showed that poly(dC) and poly(C), but not poly(dA), are effective templates for this reverse transcriptase. A template constructed from denatured HeLa DNA and an acid-soluble HeLa DNA fraction obtained after pancreatic DNase digestion appeared to be one-half as good as activated salmon sperm DNA for the γ-polymerase, though the RLV reverse transcriptase activity could not utilize it. Denatured HeLa DNA as well as native HeLa DNA (data not shown) are poor templates. We also tested a variety of natural RNA templates with the γ-polymerase. Some of these results are shown in Table II. Natural RNA templates such as HeLa nuclear RNA primed with oligodeoxynucleotides or HeLa poly(A) containing RNA primed with $(dT)_{12-18}$ were not copied by DNA polymerase γ under these conditions but were significant templates for the viral reverse transcriptases.

TABLE III: Effect of Histone f_1 on DNA Polymerase γ and RLV Reverse Transcriptase.[a]

Template	^{3}H-Labeled Substrate	γ-Polymerase Mn^{2+} (%)	γ-Polymerase Mg^{2+} (%)	RLV Reverse Transcriptase Mn^{2+} (%)	RLV Reverse Transcriptase Mg^{2+} (%)
$(dT)_{12-18}$·poly(A)	dTTP	100	100	100	100
+ f_1		510	162	346	62
Activated DNA	dTTP	32	33	1.6	2
+ f_1		24	31	5.2	1.6
$(dG)_{12-18}$·poly(C)	dGTP	87	3	42	57
+ f_1		78	3.4	114	38

[a] DNA polymerase γ and RLV reverse transcriptase activities were assayed without KCl. The assays contained in 100 μl, 2.5 μg of template, and either 0.5 mM $MnCl_2$ or 5 mM $MgCl_2$ and 0.5 μg of histone f_1 where indicated. The γ-polymerase reaction mixtures contained 40 mM potassium phosphate whereas the reverse transcriptase assays were without phosphate. All other reaction conditions were as described in legend of Table II. The quantity of protein in the assay and the 100% values are: DNA polymerase γ, 0.4 μg, 45.8 pmol (Mn^{2+} conditions), and 47.5 pmol (Mg^{2+} conditions); Rauscher leukemia virion protein, 2.2 μg, 19.1 pmol (Mn^{2+} conditions), and 4.4 μg, 610 pmol (Mg^{2+} conditions).

The above template studies with DNA polymerase γ were carried out under assay conditions optimal for the copying of $(dT)_{12-18}$·poly(A). These conditions did not permit the copying of $(dG)_{12-18}$·poly(C) or natural RNA (Table II) by this enzyme. A search was carried out for circumstances which might

TABLE IV: Effect of Potassium Phosphate on the Activity of HeLa Cell DNA Polymerases.[a]

Template	α-Polymerase		β-Polymerase		γ-Polymerase	
	Mn^{2+} (%)	Mg^{2+} (%)	Mn^{2+} (%)	Mg^{2+} (%)	Mn^{2+} (%)	Mg^{2+} (%)
$(dT)_{12-18}$·poly(dA)	100.0	100.0	100.0	100.0	100.0	100.0
+ 50 mM KPO_4	2.3	6.4	19.4	3.8	59.6	45.8
$(dT)_{12-18}$·poly(A)	2.7	0.0	53.4	0.0	59.7	46.2
+ 50 mM KPO_4	0.1	0.0	0.0	0.0	289.0	95.7
Activated salmon sperm DNA	31.2	285.9	13.9	18.4	21.8	51.7
+ 50 mM KPO_4	102.5	220.9	7.8	8.5	37.2	51.5

[a] DNA polymerase assays were carried out in the presence or absence of 50 mM potassium phosphate. The reaction mixtures contained, in a final volume of 100 μl, 50 μg of bovine serum albumin, 50 mM Tris-HCl (pH 8.5), 100 mM KCl, 0.5 mM dithiothreitol, either 0.5 mM $MnCl_2$ or 7.5 mM $MgCl_2$, 5 μg of the indicated templates, and 0.1 mM [^{3}H]dTTP (specific activity 100 cpm/pmol). In assays for α-polymerase, KCl was omitted from the reaction mixture. When activated salmon sperm DNA was used as a template, the assays contained all four deoxynucleoside triphosphates at 0.1 mM concentration. All enzymes were dialyzed before use against 50 mM Tris-HCl (pH 7.6), 50% (v/v) glycerol, and 0.5 mM dithiothreitol. Incubations were performed at 37 °C for 30 min, and the acid-insoluble radioactivity was determined as described in Materials and Methods. The 100% values represent: DNA polymerase α, 658 pmol (Mn^{2+} conditions), and 303 pmol (Mg^{2+} conditions); DNA polymerase β, 2408 pmol (Mn^{2+} conditions), and 1157 pmol (Mg^{2+} conditions); DNA polymerase γ, 102 pmol (Mn^{2+} conditions), and 143 pmol (Mg^{2+} conditions).

permit the γ-polymerase to copy the latter templates. It was found that low salt concentrations (0.04 M KPO_4 and no KCl) in the assay permitted the copying of a poly(C) template. This is shown in Table III and is in agreement with the previous report of Spadari and Weissbach (1974) for γ-polymerase, peak II. It is of interest to note that histone f_1 which is known to stimulate the activity of RLV reverse transcriptase (Modak and Marcus, personal communication) also permits the γ-polymerase to copy a poly(A) template at a fivefold faster rate. The copying of activated DNA or poly(C) is unaffected by the histone which is presumably affecting the configuration of the poly(A) template. Though the low salt conditions permitted poly(C) copying, we were unable to detect any template activity for the γ-polymerase with natural RNA under these conditions. The templates tested included HeLa messenger RNA, macrophage mRNA, rabbit globin mRNA, Rous sarcoma virus 70S RNA, and Qβ RNA with and without oligo(dT) primers.

Effect of Potassium Phosphate on the Activity of HeLa Cell DNA Polymerases. With the template $(dT)_{12-18}$·poly(A), DNA polymerase γ showed the highest activity in the presence of 50 mM potassium phosphate and 100 to 150 mM KCl as demonstrated in Figure 3. Chang and Bollum (1973) reported that high phosphate concentrations inhibit the activity of the calf thymus DNA polymerases α and β when $(dT)_{12-18}$·poly(dA) was used as a template. We, therefore, studied the influence of phosphate upon DNA polymerases α, β, and γ in more detail. Table IV shows the result of template studies with or without phosphate concentrations which are optimal for the γ-polymerase. In general, one observes that with Mn^{2+} or Mg^{2+}, the copying of poly(dA) by the α, β, and γ-polymerases was depressed in the presence of phosphate.

Table IV reconfirms that the template poly(A) can be copied by the β- or γ-polymerases but not by DNA polymerase α. Poly(A) utilization by the β-polymerase requires Mn^{2+} and is completely abolished by 50 mM potassium phosphate. By contrast, this concentration of phosphate stimulates the activity of the γ-polymerase fivefold with the poly(A) template in the presence of Mn^{2+}. When activated DNA is used as a template, phosphate again inhibits the activity of the β-polymerase but does not affect the γ-polymerase. It is clear from Table IV that each of the DNA polymerases shows a unique and distinct response to the three templates when assayed in the absence or presence of phosphate. Studies were also performed under lower phosphate concentrations. In presence of Mn^{2+}, the $(dT)_{12-18}$·poly(dA) reading of DNA polymerase β was stimulated about 50% when 5 mM phosphate was added to the assay mixture, whereas the $(dT)_{12-18}$·poly(A) reading was depressed to 25%.

TABLE V: Comparison of Activated DNA Copying by DNA Polymerase α, β, and γ under Optimal Assay Conditions.[a]

Enzymes	% Act. under Optimal Assay Conditions for			
		β		
	α	(−NEM)	(+NEM)	γ
α	100	58	1	6
β	78	100	80	33
γ	36	83	18	100

[a] The individual DNA polymerases were assayed under α-, β-, or γ-polymerase assay conditions. The reaction mixture used for α-polymerase conditions, contained in a final volume of 100 μl, 50 μg of bovine serum albumin, 50 mM Tris-HCl (pH 8.5), 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 25 μg of activated salmon sperm DNA, 0.1 mM dATP, dCTP, dGTP, and [^{3}H]dTTP (specific activity 1000 cpm/pmol). For β-polymerase conditions, 100 mM KCl alone or together with 5 mM *N*-ethylmaleimide (NEM) was included in the reaction mixture. The γ-polymerase assay conditions were performed as given in the legend of Table II with Mg^{2+}. Incubations were carried out at 37 °C for 20 min. The 100% values are: DNA polymerase, α, 1450 pmol; DNA polymerase β, 1254 pmol; and DNA polymerase γ, 121 pmol.

Comparison of DNA Polymerase Activities under Various Assay Conditions. To further illustrate the differences between the enzymes, the copying of activated DNA by DNA polymerases α, β, and γ in the presence of Mg^{2+} was examined using the optimal assay conditions for each of the individual enzymes with this template. Therefore, assays were performed with no added salt (α-polymerase conditions) or with 0.1 M KCl (β-polymerase conditions) or with 0.13 M KCl and 0.05 M KPO_4 (γ-polymerase conditions). The results of this experiment are presented in Table V. As demonstrated for the calf thymus

enzyme α-polymerase (Bollum, 1960),the human DNA polymerase α is also quite sensitive to high ionic strength since copying of activated DNA is decreased about 2- and 18-fold under β- and γ-polymerase assay conditions. The activity of β-polymerase, however, is stimulated by 0.1 M KCl, but is depressed about threefold when the γ-polymerase conditions are used.

Examination of HeLa Cells for Oncorna Viruses. Watson et al. (1974) have previously demonstrated the presence of oncorna virus like particles in certain HeLa cell strains. Examination of our HeLa S-3 cell line by Dr. Kenneth F. Watson, University of Montana, revealed no evidence of virus in either cell extracts or in culture fluids derived from these cells. The techniques used for viral detection in these experiments were those previously published by Watson et al. (1974).

Size of Product. The reaction products which were synthesized using $(dT)_{12-18}$·poly(A) as a template [poly(A) with a s_{20} value of 8.1] with DNA polymerase γ (0.01 μg) for 2 h at 37 °C or by incubating $(dG)_{12-18}$·poly(dC) [poly(dC) with a s_{20} value of 8.8] as template with DNA polymerase γ (0.01 μg) for 30 min at 37 °C were analyzed by alkaline sucrose density gradient centrifugation. The *s* value for synthesized poly(dT) was determined to be 8.6 and that for synthesized poly(dG) was found to be 5.1.

Discussion

The extended purification of DNA polymerase γ from HeLa cells, free of oncorna virus particles, has yielded an enzyme preparation with a specific activity of 25 000 units/mg of proteins which is 70-fold greater than that previously reported (Spadari and Weissbach, 1974). This specific activity can be compared with that of the purified human α-polymerase (7281 units/mg; Sedwick et al., 1975) or to the purified human β-polymerase which shows a specific activity of 6000–36 000 units/mg with a synthetic homopolymer template (Wang et al., 1974). The purified enzyme seems to exist as a high molecular weight structure which showed an estimated molecular weight of 160 000 (by sedimentation velocity), or 275 000–330 000 (by acrylamide gel electrophoresis or by Sephadex gel filtration). In mouse myeloma cells and in normal mouse tissues similar high molecular weight forms (243 000 to 315 000) of the γ-polymerase (DNA polymerase III) have been found by Matsukage and co-workers (1975). The differing apparent molecular weights in these experiments may be partly explained if the γ-polymerase is a markedly asymmetrical molecular or if there is aggregation of the enzyme. These molecular weights are higher than that reported by Spadari and Weissbach (1974) who found the human γ-polymerase to be 120 000 and that of Yoshida et al. (1974) who reported that a 450-fold purification preparation of calf thymus DNA polymerase γ contained two subunits of 68 000 and 63 000, respectively, as determined by sodium dodecyl sulfate-acrylamide gel electrophoresis. Since our most purified HeLa cell γ-polymerase preparations are not proven to be homogeneous, we have been unable to obtain definitive information about the subunit structure of the enzyme.

The purification procedure in this paper yields only one single peak of activity in ion-exchange chromatography which appears similar to the γ-polymerase peak II previously reported by Spadari and Weissbach (1974). The purified enzyme, after DNA cellulose chromatography, will copy a poly(A) template about 10 to 20× faster than it will an activated DNA template. The residual ability of the purified γ-polymerase to copy DNA is not due to contamination of these preparations with γ-polymerase since blocking antiserum prepared against the α-polymerase does not affect the copying of activated DNA by the purified γ-polymerase (Knopf, unpublished results).

The examination of the assay conditions needed to permit copying of synthetic ribohomopolymers has permitted a clearer understanding of the template preferences of the HeLa cell γ-polymerase. The copying of poly(A) is greatly facilitated by phosphate ions in the presence of KCl with either Mg^{2+} or Mn^{2+}. Poly(dA) loses some of its effectiveness as a template, in the presence of phosphate. On the other hand, the β-polymerase cannot copy a poly(A) template in the presence of 50 mM KPO_4 and this represents a powerful means of distinguishing these two enzymes and confirms the earlier observation of Chang and Bollum (1973) with the β-polymerase. Furthermore, the high salt conditions which are optimal for the copying of poly(A) templates suppress the ability of the γ-polymerase to copy poly(C) templates. The latter polymer can be read, however, in the presence of Mn^{2+} when KCl is removed from the assay mixtures and then the poly(C) is copied almost as well as poly(A) with these low salt conditions. These experiments explain the results of Spadari and Weissbach (1974), who could show the copying of poly(C) templates with a partially purified γ-polymerase. Thus, by manipulating the assay conditions, both the γ-polymerase and the Rauscher leukemia virus reverse transcriptase can be made to copy poly(A) and poly(C) templates with the same relative efficiencies and could not be distinguished by this criteria. Another similarity between the γ-polymerase and RLV reverse transcriptase lies in the ability of histone f_1 to stimulate the copying of poly(A) templates. The stimulation of RLV reverse transcriptase by histone f_1 was first noted by Manly (1974) and Modak (personal communication) and is also seen with the γ-polymerase. This histone effect may, of course, involve a change of the template configuration to permit easier access by the enzymes rather than direct interaction with the enzymes.

None of these studies has provided definitive information as to the possible roles of the cell DNA polymerase γ. The γ-polymerase, either purified to a high degree as in this study, or at less purified stage (3500×) (Spadari and Weissbach, 1974), has not yet been shown to copy natural RNA. Though this enzyme has been implicated in the synthesis of adenovirus DNA (Ito et al., 1975), the function of the γ-polymerase in the normal cellular DNA replication process remains unknown.

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