Nuclear and Cytoplasmic Deoxyribonucleic Acid Polymerases from Rat Nephroma Cells

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We have examined the DNA polymerases found in a rat nephroma cell line. Using DEAE- and DNA-cellulose chromatography, we have found two major cytoplasmic DNA polymerases and one major and three minor DNA polymerases from the nucleus. The enzymes were all purified, characterized, and distinguished from each other by several criteria. The enzyme require, for maximal activity, a natural or synthetic double-stranded DNA, four deoxynucleoside triphosphates, and magnesium. They are inhibited to varying degrees by sodium pyrophosphate, ethidium bromide, and p-chloromercuribenzoate.

DNA polymerase activity has been described in a variety of animal cells (1), as well as from cell lines such as HeLa cells (2), KB cells (3), and Ehrlich ascites tumor cells (4). The original studies in eukaryotic cells (5, 6) attempted to isolate a single DNA polymerase and to characterize the polymerase in terms of the well defined properties of Escherichia coli polymerase I (7). In the past few years, it has become known that in eukaryotic cells, as in prokaryotic cells (8-11), there are multiple DNA polymerases. A single DNA polymerase has been reported in the cellular cytoplasm and one or two distinct DNA polymerases have been located in the nucleus (12-17). In addition animal cells contain a DNA polymerase associated with the mitochondria (18-20) and an RNA-dependent DNA polymerase (R-DNA polymerase) which is distributed in the nucleus and cytoplasm (21).

The rat nephroma cells used in our present studies were first cultured in 1967 and have been maintained as a continuous cell line (22). The cells grow rapidly in monolayer cell cultures and have been used to support the growth of both human and animal viruses. We have used the cell line to grow the parvovirus, Kilham rat virus. This virus has an associated DNA polymerase activity and we hoped that characterization of the DNA polymerases from the cell and the virus (23) might lead to a better understanding of the role of these enzymes in the growth of the virus and the resulting lysis of the cell. We have found that in the rat nephroma cell the cytoplasm contains two distinct DNA polymerase activities. The cell nucleus contains one major DNA polymerase and several minor DNA polymerase activities. All of the enzymes were purified by the same chromatographic procedures. The enzymes can be distinguished from each other on the basis of chromatographic and enzymatic characteristics.

EXPERIMENTAL PROCEDURE

Materials

3H-labeled deoxynucleotides and ribonucleotides were obtained from New England Nuclear Corp. Unlabeled nucleotides and salmon sperm DNA were obtained from Calbiochem; pancreatic DNaase I and micrococcal nuclease from Worthington; poly(dA-T) and poly(dG-C) from Miles Laboratories; DEAE-cellulose (DE52) and phosphocellulose (P11) from Whatman. Munlstall 110 cells were purchased from Bio-Rad. The molecular weight standards, bovine liver catalase (50,000 units) and Escherichia coli alkaline phosphatase (36 units/mg), were purchased from Worthington Biochemical Corp. Bovine serum albumin was obtained from Mann Research Laboratories. Myoglobin was purchased from Sigma. (dT)₉, (A)₉ was obtained from A. Weissbach, Roche Institute of Molecular Biology.

Methods

Growth of Rat Nephroma Cells

The cell line used in these studies was obtained from Virginia Babcock (22). The cells were grown as monolayers in Eagle's basal medium containing 0.05% glutamine and supplemented with 10% fetal bovine serum (Grand Island Biological Co.). The rapidly dividing cells were harvested before they became a confluent cell monolayer. The cells are believed to be free of contaminating viruses, although latent viruses cannot be rigorously excluded. The uninfected cell does not spontaneously lyse or produce plaques detected by our agar plaquing procedure (24). Extracted, uninfected cells pulse-labeled with 3H-thymidine do not produce radioactive bands attributable to virus that can be detected by isopycnic sedimentation in CsCl or velocity sedimentation in sucrose.

Nuclease Treatment of Primer-Template DNA

Activated salmon sperm DNA was prepared with pancreatic DNaase I under conditions which rendered 20% of the DNA acid-soluble (17). The DNA solution was then dialyzed against 10 mM Tris-HCl, buffer, pH 7.4, to eliminate Mg 2+ ions and small nucleotides. Heat-denatured DNA was prepared by heating a DNA solution at 100°C for 10 min followed by immediate chilling in ice. Micrococcal nuclease, which catalyzes the hydrolysis of deoxyribonucleic acid to produce 3'-deoxynucleotides, was used as described in the Worthington Enzyme Manual.

Assay of Rat Nephroma Cell DNA Polymerases

Incubation mixtures contained in 200 μl Tris-HCl, 20 mM, at the optimal pH stated under "Results"; bovine serum albumin Fraction V, 5 μg; β-mercaptoethanol, 20 mM; activated salmon sperm DNA, 120 μg; dCTP, dGTP, dTTP, 50 nM each; [3H]dATP, 0.5 μCi with a specific activity of 0.5 mCi/μmol. MgCl₂ and KCl were added at the optimal
concentration for each enzyme stated in the results. The assays were carried out for 60 min at 37°C. The DNA was precipitated in 5% trichloroacetic acid at 4°C; collected on a Whatman GF/C filter; washed with five aliquots of 5 ml of trichloroacetic acid, dried, and counted in 10 ml of Spectrafluor in a Beckman scintillation counter.

Assay of Rat Nephroma Polymerase Fractions for Nuclease Activity

DNA exonuclease activity was measured by the release of acid-soluble radioactivity from radioactive, linear, vacinia DNA in 1 hour at 37°C under the optimal polymerizing conditions for each enzyme but in the absence of deoxynucleoside triphosphates. DNA endonuclease activity was determined by incubation of the purified enzymes for 1 hour at 37°C with radioactive closed circular (form I) double-stranded SV40 DNA. Optimal incubation conditions were used for each enzyme but in the absence of deoxynucleoside triphosphates. The incubation mixture was then sedimented in a neutral sucrose gradient under described conditions (25) in which the conversion of form I (21 S) to form II (16 S) can be followed. This conversion is effected by a single endonucleolytic scission of one of the DNA strands (26).

Determination of Sedimentation Coefficient and Molecular Weights by Sucrose Gradient Centrifugation

The method described by Martin and Ames (27) was used to determine the sedimentation behavior of the DNA polymerase fractions. The preparations (total volume 90 μl) were layered onto a 5 to 20% sucrose gradient containing 0.1 M Tris, pH 8.4, 10 mM EDTA, 10 mM dithiothreitol, and 0.2% Nonidet P-40 and sedimented in a 50.1 rotor at 38,000 rpm for 17 hours at 4°C in a Beckman L-55 ultracentrifuge. The standards used were: bovine liver catalase (s20w = 11.3, MW = 244,000), E. coli alkaline phosphatase (s20w = 6.5, MW = 86,000), bovine serum albumin (s20w = 4.41, MW = 68,000), and myoglobin (s20w = 2.04, MW = 17,000). Alkaline phosphatase was assayed according to Garen and Levinthal (28). Catalase was measured as described by Beers and Sizer (29). Myoglobin was detected by turbidity measurements after addition of trichloroacetic acid (30).

Examination of the enzyme fractions on 7.5% Na dodecyl SO4-polyacrylamide gels was done as previously described (31).

Preparation of Column Materials

DEAE-cellulose—DEAE (DE52) was equilibrated in 60 volumes of 0.025 M potassium phosphate, pH 8.5. Two grams of bovine serum albumin and 80 g of the washed DEAE were stirred together for 1 hour at 4°C in 500 ml of 0.025 M potassium phosphate, pH 8.5. The slurry was washed with 1 volume of 0.025 M potassium phosphate, pH 8.5, for 48 hours at 4°C in 50.1 rotor. The suspension behavior of the DNA polymerase fractions was determined at 100,000 x g for 10 min to remove nuclei and cell debris and then at 10,000 x g for 15 min to sediment organelles. The supernatant (90 ml) was used as a crude extract for purification of the cytoplasmic enzyme. The crude nuclear pellet was washed in 10 volumes of 5 x 10-4 M dithiothreitol, 0.23 M sucrose, 1 x 10-4 M MgCl2, 1 x 10-4 M potassium phosphate, pH 6.8, and 5 x 10-4 M dithiothreitol for 15 min. They were broken by gentle shear in a Dounce homogenizer with the B pestle. The extract was centrifuged at 1,200 x g for 10 min to remove nuclei and cell debris and then at 10,000 x g for 15 min to sediment organelles. The supernatent (90 ml) was used as a crude extract for purification of the cytoplasmic enzyme. The crude nuclear pellet was washed in 10 volumes of 5 x 10-4 M dithiothreitol, 0.52 M sucrose, 1 x 10-4 M MgCl2, 1 x 10-4 M potassium phosphate, pH 6.8, and 5 x 10-4 M dithiothreitol, homogenized as described above, collected by centrifugation at 200 x g for 10 min, and stored at -70°C. When used, the purified frozen nuclei were thawed and suspended in 9 volumes of the sucrose buffer described above, and the solution brought to 4°C with solid NaCl and 1 x 10-2 M EDTA. The suspension was stirred at 4°C for 4 hours. The extract was then dialyzed against five changes of 90 volumes each of 0.025 M potassium phosphate, pH 8.5, for 48 hours at 4°C. The bulk of the nuclear DNA was precipitated by this procedure and was removed by centrifugation at 10,000 x g for 5 min.

Purification of Enzymes

All of the enzymes were kept at 0-4°C for the purification procedures.

Cytoplasmic Polymerases

DEAE-cellulose Chromatography—Crude cytoplasmic extracts were dialyzed against 80 volumes of 0.02 M potassium phosphate, pH 7.5, 5 x 10-3 M dithiothreitol, and 1 x 10-2 M EDTA for 16 hours with one change of dialysis buffer. A 60-ml aliquot of the extract, total volume 90 ml, was then added to a DEAE-cellulose column (18 x 2 cm) equilibrated with the above buffer. The column was washed with this buffer until all unadsorbed protein was removed and then with a 300 ml linear gradient of 0.02 to 0.5 M potassium phosphate, 5 x 10-4 M dithiothreitol, and 1 x 10-3 M EDTA. The active fractions were pooled and concentrated in dialysis tubing with aseptic saline.

DNA-cellulose Chromatography

The concentrated enzyme fractions were dialyzed against 20 volumes of 0.1% Triton X-100, 0.02 M Tris, pH 7.4, 5 x 10-4 M dithiothreitol, 10% glycerol, and 1 x 10-3 M EDTA. They were then added to individual DNA cellulose columns (9 x 1 cm) equilibrated with the same buffer and washed with this buffer until the unadsorbed protein was removed. The protein was eluted with 160 ml of a linear gradient of 0 to 1.0 M NaCl in the above buffer. The active fractions were combined and an aliquot was dialyzed against 100 volumes of the 0.1% Triton X-100/10% glycerol buffer above.

Phosphocellulose Column Chromatography

The dialyzed active enzyme fractions were added to individual phosphocellulose columns (7 x 1 cm). The column was washed with 0.02 M potassium phosphate, pH 7.4, 5 x 10-4 M dithiothreitol, 0.1% Triton X-100, 10% glycerol, and 1 x 10-3 M EDTA until the unadsorbed protein was removed. The adsorbed protein was then eluted with a 60-ml linear gradient of 0.02 to 0.5 M potassium phosphate, pH 7.4, in the above buffer system. The peak fractions were pooled and assayed. The enzymes could be stored at 4°C or at -17°C for several weeks without appreciable loss of activity.

Nuclear Polymerases

The extracted nuclear polymerases were dialyzed against 80 volumes of 0.02 M potassium phosphate, pH 7.5, 5 x 10-4 M dithiothreitol, and 1 x 10-3 M EDTA as described for the cytoplasmic polymerases. The nuclear enzymes were then added successively to a DEAE-cellulose column, a DNA-cellulose column, and a phosphocellulose column as described for the cytoplasmic polymerases. The enzymes could be stored at 4°C or at -17°C for several weeks without appreciable loss of activity.
RESULTS

Purification of Enzymes

Cytoplasmic Polymerases—When the crude dialyzed cytoplasmic extract was applied to and eluted from a DEAE-cellulose column, the polymerase activity was located in two discrete peaks (Fig. 1). The first peak of polymerase activity (C₁) was eluted with the starting buffer and contained 33% of the recovered enzyme activity. The second peak of enzyme activity (C₁₁) represented 67% of the recovered polymerase activity and was eluted with 0.1 M potassium phosphate. When the DEAE-cellulose C₁ peak was added to and eluted from a DNA-cellulose column, over 80% of the enzyme activity was eluted with 0.55 M NaCl. A small amount of polymerase activity (10 to 15%) was eluted with 0.23 M NaCl (Fig. 2). Over 95% of the activity of the DEAE-cellulose C₁₁ peak was eluted from DNA-cellulose with 0.22 M NaCl. Both of the DNA-cellulose C₁ and C₁₁ peaks of activity eluted from phosphocellulose with the starting buffer. On the basis of elution characteristics from DEAE-cellulose and DNA-cellulose columns, there appear to be two DNA polymerases in the cytoplasm of rat nephroma cells. A representative purification of these polymerases is shown in the top portion of Table I. The two cytoplasmic polymerases have been purified 600- to 800-fold in 8 to 16% total yield to specific activities of 1820 (C₁) and 2049 (C₁₁).

Nuclear Polymerases—The DNA polymerases from the nucleus were purified using the same procedure as described for the cytoplasmic enzymes. The extract from the purified disrupted nuclei was added to a DEAE-cellulose column. Two DNA polymerase activities were eluted. The first peak of activity was eluted with 0.02 M potassium phosphate and represented approximately 10 to 15% of the recovered activity (Fig. 3, N₁). The second peak of activity was eluted with 0.1 M potassium phosphate (Fig. 3, N) and represented 85 to 90% of the polymerase activity. N₁ eluted as a single peak with 0.125 M NaCl from a DNA-cellulose column. Because of the small amount of this enzyme, it was not purified further and the characterization of the enzyme was done with material eluted from the DNA-cellulose column. N₁ was purified 100 to 200 times the activity of the original crude nuclear extract. It has a specific activity of 480 (Table I). This elution pattern was reproducible on an additional preparation of the enzymes.

When the N fraction from the DEAE-cellulose column was eluted from DNA-cellulose, three peaks of DNA polymerase activity were detected (Fig. 4). The major peak of enzyme activity (N₂) was eluted with 0.2 M NaCl and accounted for 70 to 80% of the recovered activity. A small peak (N₃) of enzyme activity (4%) was eluted with 0.05 M NaCl and a larger peak (N₄) of enzyme activity (15 to 20%) was eluted with 0.45 M NaCl. The DNA polymerases N₁, N₂, and N₄ were further purified by phosphocellulose column chromatography. Each activity eluted as a single peak of activity from phosphocellulose, N₂ at 0.05 M potassium phosphate, and N₃ and N₄ at 0.11 M potassium phosphate. N₁, because of limiting amounts, was purified only to a specific activity of 130. N₂ was purified to a specific activity of 2575, and N₃ was purified to a specific activity of 343 (Table I). The characterization of each nuclear enzyme was done with the material eluted from the phosphocellulose columns.

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Fig. 1. DEAE-cellulose chromatography of the cytoplasmic DNA polymerases from the rat nephroma cell. A 60-ml aliquot of dialyzed crude cytoplasmic extract was added to a DEAE-cellulose column (18 × 2 cm). The enzyme activity was eluted with a 300-ml linear gradient of 0.02 to 0.5 M KPO₄ (pH 7.5). The gradient elution started with Fraction 18. C₁ (Fractions 21 to 33) eluted at 0.02 M KPO₄ and C₁₁ (Fractions 41 to 47) eluted with 0.1 M KPO₄. A 0.05-ml aliquot was assayed from each fraction using the standard assay conditions described under “Methods”. Five-milliliter fractions were collected.

Fig. 2. DNA-cellulose chromatography of the cytoplasmic polymerases C₁ and C₁₁. The dialyzed DEAE-cellulose eluate fractions C₁ and C₁₁ were added to individual DNA-cellulose columns (9 × 1 cm). The protein was eluted with 160 ml of a linear gradient of 0 to 1.0 M NaCl. C₁ (Fractions 30 to 33) eluted with 0.55 M NaCl and C₁₁ (Fractions 19 to 26) eluted with 0.22 M NaCl. The gradient elution started with Fraction 15. Fractions of 3.5 ml were collected. A 0.05-ml aliquot from each fraction was assayed using conditions described under “Methods”.

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TABLE I

Purification of rat nephroma DNA polymerases

| Purification step       | Volume | Total protein | Total activity | Activity recovered from homogenate | Specific activity |
|-------------------------|--------|---------------|----------------|-------------------------------------|------------------|
| Cell homogenate         | 60 ml  | 520.0 mg      | 1520 units     | 92 units/mg protein                 | 2.9              |
| Dialyzed cytoplasm      | 60 ml  | 130.0 mg      | 1058 units     | 81.4 units/mg protein              | 5.4              |
| DEAE-cellulose peaks    |        |               |                |                                     |                  |
| C_1                     | 13.5   | 25.2          | 318 units      | 20.9 units/mg protein              | 12.6             |
| C_11                    | 18.0   | 71.0          | 652 units      | 43.0 units/mg protein             | 9.2              |
| DNA-cellulose peaks     |        |               |                |                                     |                  |
| C_2                     | 5.0    | 0.1           | 103 units      | 6.7 units/mg protein              | 1090             |
| C_11                    | 8.0    | 0.12          | 153 units      | 10.0 units/mg protein             | 1274             |
| Phosphocellulose peaks  |        |               |                |                                     |                  |
| C_1                     | 1.4    | 0.024         | 44 units       | 2.9 units/mg protein              | 1820             |
| C_11                    | 2.8    | 0.024         | 49 units       | 3.2 units/mg protein              | 2049             |
| Nuclear extract         | 2.0    | 168.0         | 356 units      | 2.3 units/mg protein              | 2.2              |
| DEAE-cellulose peaks    |        |               |                |                                     |                  |
| N_1                     | 12     | 4.5           | 130 units      | 8.4 units/mg protein              | 4.8              |
| N_2                     | 15     | 47.0          | 1171 units     | 77.2 units/mg protein             | 24.9             |
| DNA-cellulose peaks*    |        |               |                |                                     |                  |
| N_1                     | 3      | 0.25          | 120 units      | 7.5 units/mg protein              | 480              |
| N_2                     | 8      | 0.8           | 34 units       | 2.3 units/mg protein              | 43               |
| N_3                     | 15     | 1.3           | 582 units      | 38.4 units/mg protein             | 448              |
| N_4                     | 9.3    | 0.89          | 191 units      | 12.6 units/mg protein             | 205              |
| Phosphocellulose peaks  |        |               |                |                                     |                  |
| N_1                     | 0.8    | 0.01          | 1.3 units      | 0.1 units/mg protein              | 130              |
| N_2                     | 3.7    | 0.108         | 55.6 units     | 18.5 units/mg protein             | 2575             |
| N_3                     | 3.4    | 0.101         | 34.4 units     | 2.3 units/mg protein              | 343              |

*One unit is defined as 1 nmol of deoxynucleoside triphosphate incorporated into an acid-insoluble form in 60 min at 37°C.

Properties of Rat Nephroma Polymerases

General Requirements—A summary of the elution properties of the rat nephroma enzymes from DEAE-cellulose, DNA-cellulose, and phosphocellulose and some of the enzyme characteristics are shown in Table II. The two cytoplasmic enzymes, C_1 and C_11, as well as the nuclear enzymes N_1 and N_2, have identical pH optima of pH 8.9. N_1 had a pH optimum of 7.1 and had 2 to 3 times more DNA polymerase activity at this pH when compared to pH 8.9. N_2 had twice as much DNA polymerase activity at pH 7.7 than at pH 8.9. The divalent cation requirements of the enzymes were similar with the exception of the C_1 enzyme, which had a sharp Mg_2+ optimum at 0.5 to 1 mM magnesium. At 5 mM magnesium, the C_1 enzyme had only 20 to 25% of the activity present at the optimal concentration. The K_m of dNTP and the K_m of DNA were similar for all the enzymes studied. The effect of several chemical inhibitors on the activities of the rat nephroma polymerases is presented in Table III. As is generally true for the DNA polymerases, pyrophosphate inhibited all of the enzymes. The enzymes were all inhibited to varying degrees by ethidium bromide. Enzyme C_1 is very sensitive to 0.025 mM p-chloromercuribenzoate whereas the other enzymes retain 57 to 84% of their activity. Cytoplasmic enzyme C_11 is stimulated by the presence of NaCl and KCl whereas C_1 is strongly inhibited by the addition of these salts. N_1, N_2, and N_3 were unaffected by the addition of KCl and only slightly inhibited by NaCl whereas N_4, like C_1, was inhibited by both salts.

For all of the enzymes magnesium was the divalent cation which resulted in optimal enzymatic activity and produced the greatest stimulation of activity at the optimal concentration. Both manganese and calcium ions at several different concentrations resulted in a decrease in optimal enzyme activity. They do not appear to support enzymatic activity to the same extent as magnesium ions. C_11 was tested in both the absence and presence of potassium chloride. The stimulation of activity by potassium chloride was true only in the presence of magnesium ions and could not be demonstrated with manganese or calcium ions.

DNA Primer-Template Requirements—None of the rat nephroma DNA polymerases incorporates dNTP in the absence of exogenous DNA primer-template. The ability of the enzymes to use various templates is shown in Table IV. The
Some characteristics of rat nephroma DNA polymerases

The assays were carried out as described under "Methods." The DNA template was activated salmon sperm DNA.

| Table II
| DNA template requirements of rat nephroma DNA polymerases

| Primer-template | Radioactive dNTP | Cytoplasmic polymerases | Nuclear polymerases |
|-----------------|------------------|-------------------------|---------------------|
|                 |                  | I | II | 1 | 2 | 3 | 4 |
| DNAse I-treated native DNA | [3H]dATP | 100 | 100 | 100 | 100 | 100 | 100 |
| Native DNA      | [3H]dATP        | 12 | 160 | 50 | 72 | 77 | 190 |
| Denatured DNA   | [3H]dATP        | 1 | 9  | 1  | 2  | 33 | 49 |
| Denatured DNAse I-treated native DNA | [3H]dATP | 3 | 5  | 3  | 2  | 5  | 1  |
| Micrococcal nuclease-treated native DNA | [3H]dATP | 8 | 0  | 5  | 0  | 3  | 20 |
| Poly[d(A-T)]    | [3H]dATP        | 25 | 450 | 500 | 900 | 900 | 800 |
| Poly[d(A-T)]    | [3H]dTTP        | 10 | 500 | 800 | 2000 | 1800 | 2000 |
| Poly[dG-dC]     | [3H]dTTP        | 33 | 250 | 150 | 30  | 30  | 50  |
| Poly[dG-dC]     | [3H]dTTP        | 12 | 100 | 80  | 56  | 60  | 0   |

**TABLE III**

Effect of inhibitors on rat nephroma DNA polymerases

Standard assay conditions were used with the additions indicated in the table. In experiments testing the effects of p-hydroxymercuribenzoate, β-mercaptoethanol was omitted from the incubation mix. The DNA template was salmon sperm DNA. The final volume of each reaction mix was 200 μl. The assays were performed with 0.2 μg of polymerase C, 0.3 μg of polymerase N, and 0.74 μg of polymerases N and N. Activity (100%) for polymerase C was 0.31 nmol/hour; for polymerase C, 0.35 nmol/hour; for polymerase N, 3.0 nmol/hour; N, 0.041 nmol/hour; N, 0.38 nmol/hour; N, 0.25 nmol/hour.

| Addition | Cytoplasmic enzymes | Nuclear enzymes |
|----------|---------------------|-----------------|
|          | I | II | 1 | 2 | 3 | 4 |
| Standard assay | | | | | | |
| Sodium pyrophosphate, 5 mM | 100 | 100 | 100 | 100 | 100 | 100 |
| Ethidium bromide, 0.20 mM | 6 | 8 | 28 | 58 | 70 | 13 |
| p-chloromercuribenzoate, 0.025 mM | 3 | 63 | 78 | 84 | 57 | 80 |
| NaCl, 0.125 M | 18 | 134 | 63 | 70 | 33 | 100 |
| KCl, 0.1 M | 38 | 165 | 100 | 100 | 34 | 100 |

DNase I-treated DNA (activated DNA) is used as a standard of comparison for the other natural and synthetic DNAs. Magnesium was used as the divalent ion in the concentration found optimal for DNase I-treated native DNA. C, N, N, and N show greater enzyme activity with the activated DNA as opposed to native DNA. However, C, I and N, show more activity with the native DNA than with activated DNA. N, and N had reduced activity (33 to 49%) with denatured DNA, while the activity of the other enzymes is strongly reduced (less than 10%) when denatured DNA is offered as a template. Denatured DNAse I-treated DNA was a poor template-primer for all of the enzymes studied. Digestion of native DNA with micrococcal nuclease reduced its template activity for all of the enzymes demonstrating the requirement of the polymerases for 3'-hydroxyl termini.

The rat nephroma DNA polymerases differ in their ability to use synthetic primer-template as measured by the incorporation of one radioactive dNTP in the presence of its nonradioactive complement (Table IV). The cytoplasmic polymerase C uses poly[d(A-T)] poorly, and the other DNA polymerases show a great stimulation in activity with poly[d(A-T)] as a template when either [3H]dATP and dTTP or [3H]dTTP and dATP are added to the enzyme incubation mixture. The cytoplasmic polymerase C and the nuclear polymerases N, N, and N all use poly[dG-dC] with less efficiency than activated DNA whether dCTP or dGTP is supplied as a radiisotope. Cytoplasmic polymerase C and nuclear polymerase N, both use poly[dG-dC] more efficiently with [3H]dTTP than when [3H]dCTP is the isotope supplied. None of the enzymes will incorporate dCTP or dGTP with poly[d(A-T)] under the conditions used here, thus demonstrating their fidelity in copying the primer-template.

**Requirement of Rat Nephroma DNA Polymerases for Deoxynucleoside Triphosphates—**Eukaryotic DNA polymerases show maximal activity with the four deoxynucleoside triphosphates present but show substantial incorporation when only a single deoxynucleoside triphosphate is present. This was found to be true with all of the rat nephroma DNA polymerases we tested. We studied the kinetics of incorporation of one deoxynucleoside triphosphate and four nucleoside triphosphates. At all the time periods observed the incorporation of one deoxynucleoside triphosphate was less than 50% of that found with four deoxynucleoside triphosphates present. Under the usual 60 min of incubation, the extent of incorporation of one deoxynucleoside triphosphate (dATP) versus four deoxynucleoside triphosphates was C, 30%, C, 20%, N, 33%, N, 43%, N, 48%, and N, 38%.

**Sedimentation Coefficient and Molecular Weight—**An estimate of the sedimentation coefficients (s20,w) and molecular weights of the purified DNA polymerases was obtained by the method of Martin and Ames (27). The phosphocellulose fractions were used for molecular weight estimation with polymerases C, C, N, N, and N. For polymerase N, the DNA-cellulose fraction was used. Recovery of C, enzyme...
activity was 57% C-2, 88%, N, 65%, N, 51%, N, 48%, and N, 50%. The approximate calculated values for the s values and molecular weights of the rat nephroma enzyme are: C, s = 8 to 9, MW = 90,000 to 100,000; C, s = 4 to 5.5, MW = 58,000 to 70,000; N, s = 3.5 to 4, MW = 45,000 to 50,000; N, s = 8 to 9, MW = 90,000 to 100,000; N, s = 2.6 to 3.5, MW = 30,000 to 45,000. N, s = 5.5 to 6.5, MW = 70,000 to 80,000. All of the enzymes studied had, in addition to the above major peaks of enzyme activity, a small fast moving component with DNA polymerase activity and a molecular weight of 110,000 to 150,000. We do not know if this faster sedimenting band represents an aggregation of the enzyme into a complex of monomer units or a complex of more than one enzyme. All of the preparations had two to four protein bands on Na dodecylSO4-polyacrylamide gels.

**Other Enzyme Activities**—The purified DNA polymerases from the rat nephroma cell line were examined for endonuclease activity by the ability of the enzymes to effect a single endonucleolytic scission of one of the DNA strands of a supercoiled molecule of SV40 DNA. Exonuclease activity was measured by the ability of the polymerases to release acid-soluble radioactive activity from radioactive linear double-stranded vaccinia DNA preparations. Evidence for endonuclease activity was found associated only with enzyme N, which converted 57%, and with N, which converted 15% of SV40 type I molecules to type II. Evidence for exonuclease activity was found in C, N, N, and N,. However, this was at a low level, less than 2% of the polymerizing activity. Ultrasensitive assays for 3' → 5' exonuclease activity have not been carried out. Since the DNA polymerase preparations were not purified to homogeneity, it is possible that the nuclease activities are contaminating enzymes.

Attempts to demonstrate terminal deoxynucleotidyltransferase activity with all of the enzymes under optimal conditions were unsuccessful. They did not show this activity when oligonucleotides such as poly(A) or d(pT), were used as primers (33). The enzymes did not incorporate ribonucleotides in the presence or absence of deoxyxynucleoside triphosphates using the optimal conditions found for each enzyme for DNA polymerization.

Each enzyme was also incubated for 60 min under its optimal conditions using activated DNA as a template, and then the product was treated with pancreatic DNase I or pancreatic ribonuclease. In all cases the enzyme product was degraded by DNase I with release of acid soluble radioactivity but not by pancreatic ribonuclease.

**DISCUSSION**

We have studied the DNA polymerase activity found in the cytoplasmic and nuclear fractions of a rat nephroma cell line. In the cytoplasmic fraction there seem to be two major peaks of enzyme activity, C, and C,. In the nuclear fraction of the cell, DNA polymerase activity is found in one major peak, N, and three minor peaks, N, N, and N,. The enzymes were purified by chromatographic procedures but each still contains at least two bands of protein as demonstrated by polyacrylamide gel electrophoresis. In the following discussion, to the extent our data permit, we will point out the relationship of these enzymes to each other and to several prototypic DNA polymerases reported in appropriate subcellular fractions of a variety of eukaryotic cells (1-4).

Some of the properties of the rat nephroma DNA polymerases are similar to those described in other in vitro studies of DNA polymerases in eukaryotic cells (12, 16, 17). The rat nephroma cellular DNA polymerases all require some double-stranded structure for optimal priming activity. Heat denaturation of native or DNase I-treated native DNA reduces the effectiveness of the primer by at least 50%. The enzymes all require a 3’-hydroxyl terminus on the primer for activity. In the absence of the 3’-hydroxyl terminus, enzyme activity is reduced by at least 80%. The enzymes can all utilize dATP in place of dATP as a substrate for polymerization, thus demonstrating nucleoside diphosphokinase activity found in eukaryotic cellular DNA polymerases.

All of the DNA polymerases studied here with the exception of C, showed increased polymerase activity with poly[d(A-T)] as template when compared to an equal weight of activated DNA. This preference for the synthetic template has been reported previously (34-37), although it is not a characteristic property of the KB or HeLa cell DNA polymerases (16, 17).

The rat nephroma cytoplasmic enzyme, C, can be distinguished from the other nephroma cytoplasmic and nuclear DNA polymerases on the basis of its requirements for optimal enzyme activity. The enzyme activity of C, is strongly inhibited by sodium or potassium chloride. Salt does not influence the enzyme activity of C, N, N, and N,. C, has a lower magnesium ion optimum (1 mM) than the other cellular DNA polymerases (5 mM). The enzyme activity of C, is inhibited 60% in the presence of 0.5 mM magnesium chloride. C, is the only cellular enzyme with greater activity using activated DNA as a primer template than with the synthetic polymer [d(A-T)].

The DNA polymerase, C, has a sedimentation coefficient of 6 to 8S. Its sedimentation coefficient, inhibition by high salt and p-chloromercuribenzoate reduced enzyme activity with pol[y(dA-T)] or pol[y(dG-dC)] as a template are all properties shared with the cytoplasmic D-DNA polymerase II or “maxipolymerase” described in other eukaryotic cells (12, 14-17).

The cytoplasmic fraction of the rat nephroma cell contains another enzyme with DNA polymerase activity (C,). The activity of the cytoplasmic C, enzyme, like C, is inhibited at least 90% by 5 mM sodium pyrophosphate and 0.02 mM ethidium bromide. Unlike C, N, N, and N,, N, can utilize native sperm DNA as a primer template in preference to activated DNA. C, shares this characteristic with the nuclear enzyme N,. The activity of the C, enzyme is stimulated by the addition of KCl or NaCl. The stimulation of enzyme activity by salt has been reported as a property of the mitochondrial DNA polymerase (18-20) and the RNA-directed DNA polymerase (21). The RNA-directed DNA polymerase (R-DNA polymerase) resembles the DNA-directed polymerase in all of its basic requirements but is unique in the cell in its ability to utilize the ribo strand of d(T)2(A)2 to polymerize thymidylic acid. Using the conditions described by Fridlender et al. (21), the cytoplasmic enzyme C, was the only rat nephroma cellular enzyme which was able to copy the polyribomeric strand of the oligomer homopolymer complex d(T)2(A)2. C, copied the polyribomeric strand with an efficiency 1.5 to 2 times that of any other template primer, including activated DNA. Like the other R-DNA polymerases in eukaryotic cells, the rat nephroma C, enzyme did not copy any natural RNA at a significant rate (20, 21).

The major cellular DNA polymerase, N, isolated from rat nephroma cells accounts for approximately 70% of the total nuclear DNA polymerase activity. N, has been purified extensively and has a molecular weight of about 35,000 to 45,000. N, is similar in its sedimentation coefficient, molecular weight,
and requirements for optimal enzyme activity to the D-DNA polymerase I or "minipolymerase" reported in the nucleus of several cell lines and calf thymus cells (12, 14, 17).

We have found in the rat nephroma cell line three additional nuclear enzymes, N1 representing 10 to 20%, N2 3%, and N4 14% of the total nuclear DNA polymerase activity. N4 has a molecular weight of approximately 45,000, N2 of 100,000 and N4 of approximately 80,000. The rat nephroma nuclear enzymes were separated from each other by DEAE-cellulose and DNA-cellulose chromatography. They vary from each other in pH optima, in their ability to use synthetic, native and denatured DNA templates, and in their inhibition by KCl. It is not known if the nuclear enzymes are separate entities or if they are related in some way and share subunit structures. DNA polymerases N2 and N4 also may be associated with them an endonuclease activity. After a 60-min incubation with radioactive SV40 component I, 57% of the component I with enzyme N4 and 15% of component I with enzyme N2 sedimented in a sucrose gradient with the nicked circular DNA of SV40 component II. None of the other DNA polymerase fractions demonstrated detectable endonuclease activity using the method of detection. Since N2 and N4 have been shown to contain Na dodecyl- SO₄-polyacrylamide gel electrophoresis to contain at least two protein bands, it is possible that the nuclease activities are contaminating proteins. We do not know if any of the rat nephroma nuclear enzymes N1, N2 or N4 are similar to a second nuclear enzyme reported in culture human cells. The second nuclear enzyme in HeLa and KB cells has been reported to have a high molecular weight of about 100,000 but there is some disagreement whether this is a distinct entity or is related to the cytoplasmic D-RNA polymerase II (38, 39).

We have reported here two cytoplasmic and one major nuclear enzymes with DNA polymerase activity. Since breakage of cells can lead to an artificial distribution of the enzymes in various cell fractions, we feel that the intracellular location of these enzymes is not yet settled. It is possible that one or more of the polymerase activities may be due to the presence in the extract of polymerases associated with mitochondria or other subcellular constituents. However, the number of DNA polymerases isolated and the fact that the N peak appears to contain three enzymatically active fractions has made us wonder whether the enzymes are not part of a multienzyme repair or replicative system and the individual enzyme fractions may be fragments or combinations of subunits generated during the isolation procedure. We hope to explore these possibilities in future experiments.

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