Purification and Properties of Nuclear and Cytoplasmic Deoxyribonucleic Acid Polymerases from Human KB Cells*

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W. DAVID SEDWICK,† TERESA SHU-FONG WANG, AND DAVID KORN

From the Department of Pathology, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

We describe the properties of three DNA polymerases isolated from nuclear and cytoplasmic fractions of freshly harvested KB cells. Two of the enzymes, cytoplasmic polymerase and nuclear polymerase N1, have been highly purified, one of them to apparent homogeneity. The third enzyme, nuclear polymerase N2, which is only partially purified, resembles the cytoplasmic enzyme in its physical properties but possesses distinctive enzymatic characteristics that suggest it may represent an independent third protein. The KB enzymes initiate polymerization at 3'-hydroxyl termini and resemble Escherichia coli DNA polymerase II in their obligatory requirement for gap-containing templates. None of the enzymes can copy the ribonucleotide strand of hybrid templates, even under specific conditions in which E. coli DNA polymerase I does demonstrate such activity. They can, however, with low efficiency copy a deoxynucleotide strand using an oligoribonucleotide as primer. Elucidation of the possible in vivo roles of these enzymes in DNA replication, recombination, and repair must await further biochemical and genetic studies.

At present our understanding of the enzymatic basis of DNA replication in prokaryotic or eukaryotic cells is incomplete. Previous concepts, centered on the role of Escherichia coli DNA polymerase I, have been altered by the discovery of the polA mutation (1), by failure to detect changes in polymerase I activity in various DNA* mutants at restrictive temperatures, and by the recognition that cells of E. coli contain at least two additional DNA polymerases, II and III (2-5). Although evidence has been presented that implicates each of the latter two enzymes in in vivo DNA replication (6, 7), the relationships of the three recognized E. coli DNA polymerases to the putative DNA replicase remain to be established.

Since the initial description of a DNA polymerase activity in calf thymus (8, 9), studies in eukaryotic cells, influenced by prokaryote models, have been directed toward the isolation of ostensibly single, although only partially purified enzymes that have been characterized in terms of the several well defined properties of homogeneous E. coli polymerase I (10). In recent years, however, the existence of multiple DNA polymerase activities has been recognized in eukaryotic cells. These include an activity that appears to be associated with mitochondria (11, 12) and, variously, two or three separable activities in nuclei and cytoplasm (13-16). In general, and particularly with respect to human cells, none of these activities has been highly purified and its properties rigorously established.

We have previously described (17) a partially purified DNA polymerase from human KB cells, several of whose properties were significantly different from those either of E. coli polymerase I or of the calf thymus enzyme. We now report the isolation and characterization of three distinct DNA polymerases from KB cells, two of which appear to reside in the nucleus and one in the cytoplasm. Two of these enzymes have been highly purified, one of them to apparent homogeneity.

EXPERIMENTAL PROCEDURE

Materials

Unlabeled deoxyribonucleotides were obtained from P.L. Biochemicals; tritium-labeled deoxyribonucleotides and ribonucleotides from Schwartz; salmon sperm DNA from Calbiochem; pancreatic DNase I and micrococcal nucleases from Worthington; poly[d(A-T)] and poly(A) from Miles Laboratories; DEAE-cellulose (DE-52) and phosphocellulose (P11) from Whatman; Sephadex G-200 from Pharmacia; hydroxyapatite from BioRad; and ampholine carrier ampholytes from LKB. E. coli exonuclease III (Sephadex G-100 fraction (18)) was provided by I. R. Lehman; E. coli DNA polymerase I (Sephadex G-100 fraction 7 (18)) and poly[d(T)am] by D. Brutlag; poly[d(T)_{9000}] by P. Modrich, Stanford Medical School; oligo[d(T)_{15-15}] by S. Aaronson, N.I.H. and SV40 supercoiled DNA by D. A. Clayton, Stanford Medical School.

Methods

Growth of KB Cells

The cell line used in these studies was obtained from Dr. Maurice Green, St. Louis University. Cells were grown in suspen-
Nucleic Acid Purification

DNA exonuclease activity was measured as previously described (20) under standard polymerization conditions but in the absence of deoxyribonuclease triphosphates. DNA endonuclease activity was assayed by a method (21) that involves the determination by electron microscopy of the conversion of covalently closed circular molecules (Form I) of SV40 DNA to linear molecules (Form II) and 1.5 to 1.7 units of the most purified KB polymerase fractions. Incubations were carried out for 15 min at 37° and then chilled. The reaction mixtures were immediately spread for electron microscopic examination and grids prepared as previously described (22). About 150 DNA molecules were scored in each assay. Persistence of the initial percentage of Form I molecules indicates the absence of endonuclease activity.

Preparation of Column Materials

DEAE (DE-52) was equilibrated by suspension three times in 20 volumes of 0.025 M or 0.05 M potassium phosphate, pH 8.5. Thirty grams (wet weight) of equilibrated DE-52 were stirred with 1 g of bovine serum albumin in 10 volumes of one of the above phosphate buffers for 1 hour at 4°. The slurry was poured into a column and washed with 1 x 10^3 M potassium phosphate, pH 7.2, containing 20% ethylene glycol.

Hydroxyapatite—Hydroxyapatite was equilibrated with 0.05 M potassium phosphate, pH 7.2, adsorbed with bovine serum albumin as described above, and then re-equilibrated in 0.05 M potassium phosphate, pH 7.2.

Sephadex G-200—A column (2.5 x 28 cm) was prepared as described in the Pharmacia Gel Filtration Manual, and equilibrated with 0.1 M potassium phosphate, pH 8.5, containing 20% ethylene glycol. The Sephadex G-200 column was washed with 1 g of bovine serum albumin per 20 ml of column bed volume.

Other Methods

Denatured DNA was prepared by heating DNA in 0.02 M NaCl for 10 min at 100° and quenching in an ice bath. Protein was determined by a modification of the Lowry method (24); spectrophotometrically (25); or, for the most purified fraction of nuclear polymerase I, by estimation of Coomassie brilliant blue (R-250) stain intensity in SDS-acrylamide gels. Vertical slab SDS polyacrylamide gel electrophoresis was carried out according to published methods (26, 27). We are indebted to Dr. P. Greene for introducing us to this technique.

RESULTS

Purification of Enzymes

All operations were carried out at 0-4°. All buffers contained 1 x 10^{-2} M EDTA and 1 x 10^{-4} M β-mercaptoethanol, and those used in procedures after DEAE-chromatography contained in addition 20% ethylene glycol. Enzyme fractions from column steps were routinely concentrated 10-fold by dialysis against solid sucrose at 4°.

Preparation of KB Cell Extracts—Freshly harvested KB cells were suspended for 10 min in 9 volumes of hypotonic solution containing 1 x 10^{-4} M each of EDTA and β-mercaptoethanol and 2 x 10^{-3} M MgCl₂ and were then broken by gentle shear in a tight-fitting glass Dounce homogenizer with the B pestle.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; polymerase C, KB cell cytoplasmic DNA polymerase; polymerase N1, KB cell nuclear DNA polymerase 1; polymerase N2, KB cell nuclear DNA polymerase 2; dNTPs, deoxyribonucleotides.

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Breakage was >98% complete as monitored by phase microscopy. The extract was centrifuged at 1,200 g for 10 min to remove nuclei and cell debris, and then at 10,000 g for 15 min to sediment organelles. The supernatant was used as crude extract for purification of the cytoplasmic enzyme.

**Polymerase C**

**Acid Precipitation**—Crude extracts were dialyzed against 20 volumes of 0.025 M potassium phosphate, pH 6.0, for 6 hours, and the precipitate that formed in the dialysis bag was discarded.

The supernatant was further dialyzed against 20 volumes of 0.2 M sodium acetate, pH 5.5, for 4 hours. The resulting precipitate was collected by centrifugation at 10,000 g for 10 min and solubilized by homogenization into 0.2 M potassium phosphate, pH 8.5.

**Ultracentrifugation**—The solubilized pH 5.5 precipitate was adjusted to 20% (w/v) sucrose, overlayed with 5% (w/v) sucrose in the same buffer and centrifuged in the SW 40.1 rotor for 90 min at 40K rpm at 2°C. The fatty pellicles at the top of fractions were pooled and concentrated.

Enzyme fractions were either diluted with water containing 1 x 10⁻⁴ M each of MgCl₂, β-mercaptoethanol and potassium phosphate, pH 8.5, until all unadsorbed protein was removed, and then with a 150-ml linear gradient of 0.05 M potassium phosphate or dialyzed against 0.05 M potassium phosphate, pH 7.2, until all unadsorbed protein was removed. Enzyme activity was eluted either with a 150-ml linear gradient of 0.15 M to 0.40 M of the same buffer or in a single step with potassium phosphate at 0.21 M. The peak fractions of enzyme activity were pooled and concentrated. They could be stored for more than 6 months over liquid nitrogen without appreciable loss of activity.

A representative purification of polymerase C is shown in Table I, Part A.

**Nuclear Polymerses**

**Purification of Nuclei**—The crude nuclear pellet was washed with 9 volumes of buffer containing 0.3% Triton X-100, and 1 x 10⁻⁴ M each of MgCl₂, β-mercaptoethanol and potassium phosphate, pH 6.5, in a Dounce homogenizer by 10 strokes with the B pestle. The nuclei were centrifuged at 1200 g for 10 min and the supematant discarded. This procedure was repeated once. The resulting purified nuclear pellet was suspended in 0.2 M potassium phosphate, pH 8.5, and homogenized, and the centrifugation procedure repeated. The two supernatants were combined.

**DEAE-cellulose Column Chromatography**—Enzyme fractions were either diluted with water containing 1 x 10⁻⁴ M each of EDTA and β-mercaptoethanol to a final ionic strength of 0.05 M potassium phosphate or dialyzed against 0.05 M potassium phosphate, pH 8.5, and added to a DEAE-column (2.5 x 12 cm). The column was washed with 0.05 M potassium phosphate, pH 8.5, until all unadsorbed protein was removed, and then with a 150-ml linear gradient of 0.05 M to 0.2 M potassium phosphate, pH 8.5. The enzyme activity was eluted at 0.15 M. The active fractions were pooled and concentrated.

**Sephadex G-200 Gel Filtration**—The concentrated enzyme fractions were loaded on a Sephadex G-200 column (2.5 x 28 cm) (V₀ = 45 ml) in 0.15 M potassium phosphate, pH 7.2, containing 20% ethylene glycol. Elution of the column was carried out with the same buffer at a flow rate of 3.6 ml per cm² per hour. Polymerase C activity was eluted in fractions in the molecular weight range of 110,000 to 140,000, as determined by comparison with marker proteins. Peak fractions of activity were pooled and concentrated.

**Phosphocellulose Column Chromatography**—The concentrated enzyme fractions were loaded on a phosphocellulose column (2.5 x 7.5 cm), and the column was washed with 0.15 M potassium phosphate, pH 7.2, until all unadsorbed protein was removed. Enzyme activity was eluted either with a 150-ml linear gradient of 0.15 M to 0.40 M of the same buffer or in a single step with potassium phosphate at 0.21 M. The peak fractions of enzyme activity were pooled and concentrated. They could be stored for more than 6 months over liquid nitrogen without appreciable loss of activity.

**Preparation of Nuclear Crude Extract**—The purified frozen nuclei were thawed and suspended in 9 volumes of sucrose buffer to which were successively added EDTA, to 1 x 10⁻⁴ M each of MgCl₂ and β-mercaptoethanol, homogenized as described above, collected by centrifugation at 1200 g for 10 min and stored at −70°C.

### Table I

**Purification of KB cell DNA polymerases**

<table>
<thead>
<tr>
<th>Fraction or step</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Yield</th>
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<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>A. Cytoplasmic polymerase</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Crude extract*</td>
<td>433</td>
<td>4,330</td>
<td>14,263</td>
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<td>Acid precipitation</td>
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<td>1,716</td>
<td>18,262</td>
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<td>0.9</td>
<td>1,470</td>
<td>1,635</td>
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<td>B. Nuclear polymerases</td>
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<td>399</td>
<td>10,591</td>
<td>31</td>
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<td>146</td>
<td>3,115</td>
<td>21</td>
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<td>10,669</td>
<td>73</td>
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<tr>
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<td>Isoelectriefocusing</td>
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<td>0.012</td>
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<td>8,720</td>
<td>3.5</td>
</tr>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Phosphocellulose</td>
<td>74</td>
<td>27</td>
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<td>79</td>
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<td>Hydroxylapatite</td>
<td>13</td>
<td>1.8</td>
<td>1,246</td>
<td>692</td>
<td>11.6</td>
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</tbody>
</table>

* Crude extract was from 40 g of KB cells.

† Nuclear extract was from 2 x 10⁸ nuclei or approximately 100 g of KB cells.

§ In calculating yield of the nuclear polymerases, the DEAE-fractions were taken as 100%.
4° for 4 hours. The viscous extract was dialyzed for 48 hours at 4° against three changes of 10 volumes each of 0.025 M potassium phosphate, pH 8.5. The bulk of the nuclear DNA was precipitated by this procedure and was removed by centrifugation at 10,000 g for 5 min.

Polymerase N1

DEAE-cellulose Column Chromatography—Nuclear crude extract was added to a DEAE-column (2.5 × 15 cm), and the column was washed with 0.025 M potassium phosphate, pH 8.5. The fraction of added polymerase activity that did not adsorb to the column was pooled and concentrated.

Phosphocellulose Column Chromatography—The concentrated enzyme fractions were adjusted to 0.15 M potassium phosphate, pH 7.2, and loaded on a phosphocellulose column (2.5 × 7.5 cm). The column was washed with 0.15 M potassium phosphate until all unadsorbed protein was removed. Enzyme activity was eluted either at 0.25 M with a 150-ml gradient of 0.15 M to 0.40 M of potassium phosphate, pH 7.2, or in a single step at 0.25 M. Fractions with enzyme activity were pooled and concentrated.

G-200 Gel Filtration—Gel filtration was carried out as described for polymerase C. Polymerase activity was eluted in fractions of about 38,000 molecular weight as indicated by protein standards.

Isoelectric Focusing—Isoelectric focusing was carried out as described for the LKB 8101 (110 ml) column in the LKB manual. Ampholytes in the pH 8 to 10 range were used at a concentration of 2%. Polymerase N1 was focused at pH 9.2. Fractions containing polymerase activity were pooled, adjusted to 20% ethylene glycol and stored above liquid nitrogen for at least 6 months without an appreciable loss of activity.

A representative purification of polymerase N1 is shown in Table I, Part B.

Polymerase N2

DEAE-cellulose Column Chromatography—The nuclear crude extract was added to a DEAE-column (2.5 × 15 cm). The column was washed with 0.025 M potassium phosphate, pH 8.5, until all unadsorbed polymerase activity was removed, and then developed with a linear gradient of 150 ml of 0.05 M to 0.2 M potassium phosphate, pH 8.5. A peak of polymerase activity was eluted at 0.15 M. Fractions containing this enzyme activity were pooled and concentrated.

Phosphocellulose Column Chromatography—Concentrated enzyme fractions were loaded on a phosphocellulose column (2.5 × 7.5 cm). The column was washed and developed as described above for polymerase N1. Polymerase activity was eluted at 0.21 M potassium phosphate. Fractions with enzyme activity were pooled and concentrated.

Hydroxyapatite Column Chromatography—Concentrated enzyme was diluted to 0.05 M potassium phosphate, pH 7.2, and applied to a hydroxyapatite column (1.5 × 2 cm). The column was washed until all unadsorbed protein was removed. Enzyme activity was eluted as a single peak at 0.25 M by a 60-ml linear gradient of 0.05 M to 0.40 M of the same buffer. Polymerase N2 was stored over liquid nitrogen for more than 6 months without loss of activity.

A representative purification of polymerase N2 is shown in Table I, Part B.

Purity of Polymerases

Cytoplasmic Polymerase—A single DNA polymerase activity has been identified in the organelle-free cytoplasmic crude extract and has been routinely purified 500- to 1000-fold in 5 to 10% yield to a specific activity of 1600 to 2500. All of the data relating to polymerase C that are presented in this paper were obtained with the phosphocellulose fraction (Table I, Part A). Polymerase C is a more highly purified form of the major polymerase activity we previously described in frozen KB cells (17), and like that activity, neither polymerase C enzyme activity nor protein from the phosphocellulose fraction enters agarose or polyacrylamide gels under nondenaturing conditions. In SDS acrylamide gels different phosphocellulose preparations of polymerase C exhibit two to four bands in the molecular weight range of 1.1 to 1.4 × 10^6. This size is in agreement with the molecular weight estimation (1.4 × 10^6) of polymerase C enzyme activity by Sephadex G-200 gel filtration, but we have not yet established that any of the bands observed in SDS gels represent enzyme protein.

Nuclear Polymerase I—Two DNA polymerase activities have been identified in the KB nuclear extract. Polymerase N1 has been purified about 700-fold from the DEAE-fraction in 10 to 15% yield to a specific activity of 8720. The data reported herein for this enzyme were obtained with the fraction purified through the isoelectric focusing step (Table I, Part B1). The extremely small quantities of purified polymerase N1 available to date have precluded a detailed study of its physical properties. However, on slab SDS polyacrylamide gel electrophoresis, the most purified fraction exhibits a single band of molecular weight 3.9 × 10^6. This size agrees with the estimated molecular weight (3.5 × 10^6) of polymerase N1 enzyme activity obtained by gel filtration.

Nuclear Polymerase II—The second DNA polymerase activity in the nuclear extract, polymerase N2, is the least pure of the three KB enzymes. The best preparations to date (Hydroxylapatite fraction, Table I, Part B2) have a specific activity of 692, although with exonuclease III-treated DNA template the specific activity increases to 1300 (see below). This represents a purification of about 10-fold from the DEAE-fraction in 5% yield. On SDS polyacrylamide gels, the best polymerase N2 fractions contain numerous bands. Only few of the properties of the hydroxyapatite fraction of this enzyme are included for comparative purposes in this report.

Properties of KB Polymerases

General Requirements—As shown in Table II, the three KB enzymes have identical pH optima and very similar divalent cation requirements. They differ in their K_m values for deoxyribonucleoside triphosphate and strikingly in their apparent K_m values for activated salmon sperm DNA. The effects of several

| TABLE II
<table>
<thead>
<tr>
<th>Some characteristics of KB DNA polymerases</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>pH optimum</td>
</tr>
<tr>
<td>Mg^{2+} optimum</td>
</tr>
<tr>
<td>K_m of dNTP</td>
</tr>
<tr>
<td>K_m of DNA</td>
</tr>
<tr>
<td>Isoelectric pH</td>
</tr>
</tbody>
</table>

^a The buffer was Tris-chloride.

^b Enzyme activity collected in a precipitate at pH 5.6.
chemical inhibitors on the activities of polymerases C and N1 are presented in Table III. As is generally true for the DNA polymerases, pyrophosphate strongly inhibits both enzymes. Polymerase C is slightly inhibited by acridine orange and markedly inhibited by ethidium bromide, while neither of these intercalating agents affects polymerase N1 activity at the concentrations tested. Polymerase C appears to be very sensitive to p-hydroxymercuribenzoate, in contrast to the insensitivity of polymerase N1. The enzymes also differ in their response to monovalent cations, polymerase C showing profound inhibition in 0.15 M to 0.20 M salt, while polymerase N1 activity is partially stabilized by 0.20 M KCl. Thus, under standard assay conditions dNTP incorporation by polymerase N1 ceases after 30 min; in the presence of 0.20 M KCl, incorporation proceeds for up to 3 hours (see Fig. 1).

Nuclease Activities—The three KB polymerases demonstrate no detectable endonuclease activity when tested by a highly sensitive assay for nicking of supercoiled molecules of SV40 DNA (see "Experimental Procedure"). Polymerases C and N2 exhibit a low level of exonuclease activity (1 to 2% of polymerizing activity), while the most purified fraction of polymerase N1 appears to be free of exonuclease (<0.2% of polymerizing activity). However, ultrasensitive assays for 3' → 5' exonuclease activity (28, 29) have not yet been carried out. It is important to note that the gel filtration fraction of polymerase N1 (Table I, Part E1), which is at a stage of purification comparable to that reported by others (13) (i.e. 300 to 700 units per mg), contains an exonuclease activity that is equal to the polymerase activity. The nuclease activity is removed completely in the isoelectric focusing step, and we have not studied it further.

DNA Primer-Template Requirements—None of the three KB enzymes incorporates dNTP in the absence of exogenous DNA primer-template. The ability of the KB enzymes and of *E. coli* DNA polymerase I to use salmon sperm DNA after various types of modification by nucleases is shown in Table IV. In the absence of nuclease digestion, the three KB enzymes use the DNA primer-template poorly. In contrast, the *E. coli* enzyme uses the native template with 10 to 20 times the efficiency of the KB enzymes. Digestion with DNase I increases template efficiency by 40- to 100-fold for the KB enzymes, 5-fold for the *E. coli* enzyme. A similar degree of digestion with micrococcal nuclease abolishes template activity for all of the enzymes, demonstrating the absolute requirement of the polymerases for 3'-hydroxyl termini. The introduction of gaps into native DNA with exonuclease III (4, 31) produces a primer-template that is used with different degrees of facility by the different enzymes. The ratios of activity with exonuclease III template to that with DNase I template for the four enzymes are: polymerase N1, 0.14; polymerase N2, 1.9; polymerase C, 0.45; and *E. coli*, 1.1. Thus, the preference of polymerase N2 for the exonuclease III template was unique among the KB polymerases.

The KB polymerases differ dramatically from the *E. coli* enzyme in their ability to use poly[d(A-T)] as primer-template (Table IV). Polymerase C uses poly[d(A-T)] at least well, and the limited extent of incorporation is similar whether both dATP and dTTP are provided as substrates or whether either of the dNTPs is present alone. Thus, polymerase C seems able to perform only single deoxynucleotide addition to the 3'-hydroxyl termini in this copolymer. Polymerases N1 and N2 show greater ability to use this primer-template, and incorporation is 5-fold greater when both dATP and dTTP are present than when either is present alone; thus, these enzymes do appear to effect polymerization with the copolymer. It is important to emphasize that none of the KB enzymes will incorporate dCTP or dGTP with poly[d(A-T)], thus demonstrating their fidelity in copying this primer-template.

The final entry in Table IV indicates that all three KB enzymes can utilize a polynucleotide primer. Incorporation of [3H]dAMP using poly(A)-poly[d(T)50] is 10 to 45% of the incorporation observed with poly[d(A-T)]. We have not yet attempted to optimize conditions for the poly(A-dT) primer-template, and greater efficiency may be obtainable.

### Table III

*Effect of chemical inhibitors on polymerase C and polymerase N1*

Standard assay conditions were used with the additions indicated in the table. In experiments testing the effect of p-hydroxymercuribenzoate, p-mercaptoethanol was omitted from the incubation mix. The assays were performed with 2.6 μg of polymerase C and 50 ng of polymerase N1. Activity, 100%. for polymerase C was 5.2 nmoles per hour; for polymerase N1, 0.42 nmoles per hour.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Polymerase C</th>
<th>Polymerase N1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% activity</td>
<td>% activity</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>Acridine orange</td>
<td>86</td>
<td>98</td>
</tr>
<tr>
<td>20 μM</td>
<td>85</td>
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<td>50 μM</td>
<td>81</td>
<td>106</td>
</tr>
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<td>Ethidium bromide</td>
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<tr>
<td>10 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μM</td>
<td></td>
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</tr>
<tr>
<td>10 mM p-Hydroxymercuribenzoate</td>
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<td>105</td>
</tr>
<tr>
<td>50 μM</td>
<td>15</td>
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<tr>
<td>NaCl 0.15 M</td>
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</tr>
<tr>
<td>KCl 0.2 M</td>
<td>11</td>
<td>113</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Kinetics of incorporation of 4 dNTPs versus 1 dNTP by polymerase N1. The reaction mixtures contained, in a total volume of 2 ml: 10 mM Tris-HCl, pH 9.2; 2 mM β-mercaptoethanol; 200 μg per ml of bovine serum albumin; 10 mM MgCl₂; 0.2 M KCl; 120 μg of activated salmon sperm DNA and 200 ng of polymerase N1. One mixture (○—○) contained 200 μM each of dATP, dGTP, dCTP and dTTP and [3H]dTTP (0.04 Ci per mmol); the second (○—○) contained 200 μM dTTP only plus [3H]dTTP (0.04 Ci per mmol). Incubation was carried out at 37°, and 0.25-ml aliquots were removed at the indicated times for determination of [3H]dTMP incorporation. At 180 min (△) an additional 100 ng of polymerase N1 were added to each reaction vessel and incubation continued for another 180 min.
Utilization of RNA-DNA Hybrid Templates—The ability of the three KB polymerases to use hybrid templates was tested under the conditions described by Ross et al. (32), Stavrianopoulos et al. (33), and Goodman and Spiegelman (34). We used both poly(A)·poly[d(T)200] and poly(A)·poly[d(T)11-15] at appropriate pH values, with KCl, and with either Mn²⁺ or Mg²⁺ as divalent cation. Under the conditions reported to be optimal for hybrid template use, polymerization with activated DNA was poor; polymerase C, 15% and polymerase N1, 22% respectively, of the levels of incorporation observed under standard assay conditions. Reading of the polyribonucleotide strand of the hybrid was at best 0.9% (polymerase C) and 0.4% (polymerase N1) of the reduced activity observed with activated DNA under the same conditions. Thus, the highly purified polymerases C and N1 cannot use a polyribonucleotide as template. Polymerase N2 has not yet been adequately tested.

Use of Deoxynucleoside Diphosphate as Substrate by DNA Polymerases—Miller and Wells (35) have reported the presence of nucleoside diphosphokinase-like activity in E. coli DNA polymerase I. As shown in Table V, both polymerases C and N1, as well as the E. coli enzyme, possess a similar activity which is particularly well demonstrated with poly[d(A-T)]. Polymerase C, which carries out only a limited single nucleotide addition with this template, utilizes dADP as well as dATP, and incorporation is not increased by adding ATP to the incubation. Polymerase N1 uses dADP more efficiently than the E. coli enzyme, and incorporation is enhanced either by adding ATP to the reaction or by preliminary incubation of polymerase N1 with substrates and ATP in the absence of template. With activated DNA primer-template, all three enzymes show low but significant incorporation with dADP, and incorporation is stimulated 3- to 5-fold with each enzyme in the presence of ATP.

**Table IV**

<table>
<thead>
<tr>
<th>Template</th>
<th>dAMP incorporated</th>
<th>dAMP incorporated</th>
<th>dAMP incorporated</th>
<th>dAMP incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymerase C</td>
<td>Polymerase N1</td>
<td>Polymerase N2</td>
<td>E. coli polymerase I</td>
</tr>
<tr>
<td></td>
<td>pmole/hr %</td>
<td>pmole/hr %</td>
<td>pmole/hr %</td>
<td>pmole/hr %</td>
</tr>
<tr>
<td>DNase I-treated native DNA</td>
<td>697 (100)</td>
<td>605 (100)</td>
<td>776 (100)</td>
<td>662 (100)</td>
</tr>
<tr>
<td>Native DNA</td>
<td>8 (1.2)</td>
<td>5 (0.8)</td>
<td>18 (2.3)</td>
<td>155 (19.5)</td>
</tr>
<tr>
<td>Denatured DNA</td>
<td>22 (3.2)</td>
<td>22 (3.7)</td>
<td>89 (11.4)</td>
<td>28 (4.2)</td>
</tr>
<tr>
<td>Exo III-treated native DNA</td>
<td>313 (48)</td>
<td>87 (14.4)</td>
<td>1489 (192)</td>
<td>717 (108)</td>
</tr>
<tr>
<td>Micrococcal nuclease-treated native DNA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>poly[d(A-T)]</td>
<td>29 (4.2)</td>
<td>121 (20)</td>
<td>390 (50.3)</td>
<td>1136 (172)</td>
</tr>
<tr>
<td>poly(A)-poly(d[T]100)</td>
<td>13 (1.9)</td>
<td>22 (3.6)</td>
<td>46 (5.9)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table V**

Use of deoxynucleoside diphosphate as substrate by DNA polymerases

Standard incubation conditions were used for the KB polymerases and for E. coli polymerase I, respectively. Substrates, templates (100 μg per ml) and addition of ATP (1 μM) were as indicated in the table. When dADP was used as substrate in the absence of ATP, the other dNTPs were present at twice their normal concentration. The assays were performed with 500 ng of polymerase C, 25 ng of polymerase N1 and 600 ng of E. coli polymerase I, for 1 hour at 37°. The labeled substrates (dADP and dATP) were checked for purity by paper chromatography (36). The chromatograms were cut into 1-cm strips and counted in a liquid scintillation spectrometer. dATP contamination of the tritiated dADP was <0.07%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Addition</th>
<th>Template</th>
<th>Incorporation of [3H]-dAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP, dTTP</td>
<td>None</td>
<td>[d(A-T)]</td>
<td></td>
</tr>
<tr>
<td>dADP, dTTP</td>
<td>None</td>
<td>dATP</td>
<td></td>
</tr>
<tr>
<td>dADP, dTTP</td>
<td>ATP</td>
<td>Activated DNA</td>
<td></td>
</tr>
<tr>
<td>dATP, dTTP, dGTP, dCTP</td>
<td>None</td>
<td>E. coli DNA</td>
<td></td>
</tr>
<tr>
<td>dADP, dTTP, dGTP, dCTP</td>
<td>None</td>
<td>E. coli DNA</td>
<td></td>
</tr>
<tr>
<td>dADP, dTTP, dGTP, dCTP</td>
<td>ATP</td>
<td>E. coli DNA</td>
<td></td>
</tr>
</tbody>
</table>

a Preincubation was carried out at 37° for 60 min in the presence of all constituents of the assay except template. The tubes were heated at 100° for 2 min, and the polymerisation was then begun by addition of template and fresh enzyme.
of the primer-template and of the duration of incubation. Thus, with activated DNA and E. coli polymerase I, the extent of incorporation with 1 dNTP versus 4 dNTPs was 4.5% after 5 min, 2% after 10 min, and <1% after 30 min, the usual incubation time. Under similar conditions the comparable values obtained with the KB enzymes after 10 min of reaction were polymerase C, 5 to 10%; polymerase N2, 10%; and polymerase N1, 80%.

The kinetics of incorporation of 1 dNTP versus 4 dNTPs by polymerase N1 with activated DNA are depicted in Fig. 1. In contrast to E. coli polymerase I, in which incorporation of a single dNTP has reached completion by 5 min, the rate of incorporation of a single dNTP by polymerase N1 is comparable to that observed in the presence of all 4 dNTPs during the first 10 min of incubation. Thereafter, the rate of reaction abruptly declines (3 to 5% of initial rate) and is unaffected by the addition of fresh enzyme at 180 min. With 4 dNTPs, incorporation is brisk during the initial 30 min of reaction and then proceeds at a reduced but linear rate (20% of initial rate) for another 150 min. Addition of fresh enzyme at 180 min reproduces the initial reaction kinetics, and after 360 min of incubation, the extent of incorporation with a single dNTP is only 15% of that attained in the complete reaction. The biphasic kinetics suggest that a rapid rate of initiation is followed by a slower rate of polymerization, the extent of which is limited by enzyme instability that has not yet been overcome.

By incubating increasing quantities of E. coli polymerase I with a limited amount of DNA primer-template for 5 min in the presence of a single dNTP, it is possible to achieve saturating levels of enzyme and to measure the number of 3'-hydroxyl initiation sites by the extent of incorporation of the single deoxyribonucleotide (37). When the salmon sperm DNA preparations used in this report were analyzed in this way, the extent of incorporation with native DNA was 11 pmoles/25 µg of DNA (2.6 × 10^3 3'-OH sites); with exonuclease III-treated DNA, 36 pmoles/25 µg of DNA (8.6 × 10^3 3'-OH sites); and with DNAse I-treated DNA, >100 pmoles/25 µg (>2.4 × 10^4 3'-OH sites). The total amount of single nucleotide incorporated by polymerase N1 (and the other KB enzymes) never exceeded, 36 pmoles/25 µg of DNA (8.6 × 10^3 3'-OH sites) ; and with DNase I-treated DNA, > 100 pmoles/25 µg (>2.4 × 10^4 3'-OH sites) ; and with their utilization of templates, the KB enzymes resemble E. coli polymerase I (19) and II (31) in showing an apparent requirement for gaps or nicks. This conclusion with the recent HeLa cell study reported by Fridlender et al. (40). The KB enzymes differ from E. coli polymerase I in their inability to copy a polyribonucleotide strand under the assay conditions of Karkas et al. (41). Like the E. coli enzyme, both polymerases C and N1 can use dADP in place of dATP as substrate for polymerization, and utilization is enhanced in the presence of ATP. Thus, this nucleoside diphosphokinase-like activity may prove to be a general property (or contaminant) of both prokaryotic and eukaryotic DNA polymerases.

A particularly vexing problem with most reported mammalian DNA polymerases (see References 17 and 42) has been their apparent disproportionate reactivity in the presence of only a single dNTP. By measuring the number of available 3'-hydroxyl termini in our DNA primer-template, we have now clearly demonstrated that the amount of single nucleotide incorporated is always substantially less than the number of initiation sites available for single base addition. This should eliminate allegations that mammalian DNA polymerase preparations are necessarily contaminated by a terminal transferase activity that sequesters enzyme activity at nicks. Nonetheless, until the polymerase N2 activity is further purified, its identity as a unique, third KB polymerase cannot be proved. It is of interest, however, that all three KB enzymes differ significantly from a highly purified DNA polymerase from rat liver (38), suggesting that there may be distinctive differences between the DNA polymerases of human and nonhuman mammalian cells.

Like all other DNA polymerases, the three KB enzymes have an obligatory requirement for 3'-hydroxyl initiation sites. In their utilization of templates, the KB enzymes resemble E. coli polymerase I (18) and II (5). The most purified fraction of polymerase N1 contains only a single protein band of 39,000 daltons by SDS-polyacrylamide gel electrophoresis, but because of the very limited amounts of enzyme available, the coincidence of enzyme activity and protein has not yet been established in nondenaturing gels. The best preparations of polymerase C, the polymerase we have described (17) in extracts of frozen KB cells, presents two to four bands in SDS gels in a molecular weight range (110,000 to 140,000) that is consistent with that of polymerase C enzyme activity as measured by gel filtration. As we previously reported, this enzyme does not enter polyacrylamide gels under a variety of nondenaturing conditions, for reasons not yet clear.

The third KB polymerase, polymerase N2, has only been purified to a degree comparable to that described by Weissbach et al. (13). This enzyme is similar in chromatographic behavior and molecular weight (as estimated by gel filtration) to polymerase C, but our preliminary studies have revealed a number of enzymatic properties that distinguish it from the cytoplasmic polymerase. Thus, in comparison to polymerase C, polymerase N2 shows a higher affinity for DNA, preference for exonuclease III-treated DNA primer-template and relatively greater ability to use poly[d(A-T)]. Nonetheless, until the polymerase N2 activity is further purified, its identity as a unique, third KB polymerase cannot be proved. It is of interest, however, that all three KB enzymes differ significantly from a highly purified DNA polymerase from rat liver (38), suggesting that there may be distinctive differences between the DNA polymerases of human and nonhuman mammalian cells.

None of the three KB polymerases contains detectable endonuclease activity. Polymerases C and N2 have low levels of an exonuclease activity that has not been characterized, but we have not been able to detect exonuclease activity in the most purified fraction of polymerase N1 using conventional assays. Although cytoplasmic extracts of KB cells contain a hybrid template activity (unpublished experiments) similar to that recently described (32, 34) in mammalian cells, the activity is not associated with any of the KB DNA polymerases. This finding is in agreement with the recent HeLa cell study reported by Fridlender et al. (40). The KB enzymes differ from E. coli polymerase I in their inability to copy a polyribonucleotide strand under the assay conditions of Karkas et al. (41). Like the E. coli enzyme, both polymerases C and N1 can use dADP in place of dATP as substrate for polymerization, and utilization is enhanced in the presence of ATP. Thus, this nucleoside diphosphokinase-like activity may prove to be a general property (or contaminant) of both prokaryotic and eukaryotic DNA polymerases.

Finally, we note that none of the KB polymerases demonstrates activity when assayed with d(pT) under optimum conditions for terminal transferase (38), nor do they incorporate ribonucleotides in the presence of absence of dNTPs.

DISCUSSION

Several recent papers have noted the presence of distinct DNA polymerases in crude nuclear and cytoplasmic extracts of mammalian cells (13-16), and Weissbach et al. (13) have reported some of the properties of three partially purified DNA polymerases in extracts of frozen HeLa cells. The three enzymes we describe in this paper were obtained from freshly harvested human KB cells and resemble the HeLa cell polymerases in their distribution and major characteristics. The several reports are in agreement that a low molecular weight DNA polymerase can be isolated from nuclei and a high molecular weight DNA polymerase from the cytoplasm.

We have purified polymerases C and N1 to specific activities of 1600 to 2500 and 7500 to 8000, respectively, which compare favorably with those of the most highly purified preparations of E. coli DNA polymerase I (18) and II (5). The most purified fraction of polymerase N1 contains only a single protein band of 39,000 daltons by SDS-polyacrylamide gel electrophoresis, but because of the very limited amounts of enzyme available, the coincidence of enzyme activity and protein has not yet been established in nondenaturing gels. The best preparations of polymerase C, the polymerase we have described (17) in extracts of frozen KB cells, presents two to four bands in SDS gels in a molecular weight range (110,000 to 140,000) that is consistent with that of polymerase C enzyme activity as measured by gel filtration. As we previously reported, this enzyme does not enter polyacrylamide gels under a variety of nondenaturing conditions, for reasons not yet clear.

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do not hallucinate.
sion is based on the following observations: Native salmon sperm DNA is almost inactive as template, and yet, as determined with E. coli polymerase I, it contains a large number of 3'-hydroxyl termini (2.6 × 10⁹/25 μg of DNA). The template can be activated 20- to 40-fold by digestion with exonuclease III, a treatment that converts nicks to gaps, but leads to only a modest increase in 3'-hydroxyl termini (8.6 × 10⁹/25 μg of DNA). The DNA is also markedly activated by extensive digestion with DNase I (20% of the DNA rendered acid-soluble) which increases the number of 3'-hydroxyl termini >10-fold, but more significantly generates a large number of small gaps (31). The requirement for gaps most likely reflects the absence in the KB enzymes of the 5'-exonuclease activity of E. coli polymerase I (44), and inability to displace the 5'-strand ahead of the growing point in the absence of nucleolytic digestion (29). The relative preference of the three KB polymerases for DNase I-treated versus exonuclease III-treated templates varies (Table IV). The most striking discrimination is exhibited by polymerase N₁, whose preference for DNase I-treated template may reflect an inability to traverse long gaps. This raises the question of whether such gaps might be more effectively utilized in the presence of a DNA-stabilizing protein such as the phage T4 gene 32 product (45, 46) and suggests a possibly convenient assay system with which to seek such a specific protein in KB cells.

Finally, we note that all of the KB polymerases can utilize poly(A) as primer for the incorporation of dAMP on a poly(dT) template. Although we have not yet studied this type of reaction in detail, the property is noteworthy in view of recent reports linking DNA polymerization to RNA polymerization in vitro (41).

In conclusion, the property is noteworthy in view of recent reports linking DNA polymerization to RNA polymerization in vitro (41). Finally, we note that all of the KB polymerases can utilize poly(A) as primer for the incorporation of dAMP on a poly(dT) template. Although we have not yet studied this type of reaction in detail, the property is noteworthy in view of recent reports linking DNA polymerization to RNA polymerization in vitro (41).

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REFERENCES

30. Richardson, C. C., Schildevaust, C. L., Appishaw, H. V., and Kornberg, A. (1964) J. Biol. Chem. 239, 222
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W. David Sedwick, Teresa Shu-Fong Wang and David Korn


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