"Cytoplasmic" Deoxyribonucleic Acid Polymerase

STRUCTURE AND PROPERTIES OF THE HIGHLY PURIFIED ENZYME FROM HUMAN KB CELLS*

(Received for publication, January 13, 1975)

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The freshly prepared crude cytoplasmic fraction of aqueously extracted KB cells contains a single major species of DNA polymerase activity (DNA polymerase C) that sediments homogeneously in low ionic strength sucrose gradients with a peak at 10.8 S. The enzyme activity from frozen crude extracts sediments heterogeneously under these conditions with peaks at 8.4 and 10 S. In 0.45 M salt-containing gradients all of the polymerase activity is recovered as a single 6.4 S species. When purified to a specific activity of 7,300, DNA polymerase C sediments in low ionic strength gradients as a single species of 6.5 S. From combined sedimentation and gel filtration analysis, we estimate the molecular weight of the active protomeric species of the polymerase to be about 170,000. Under no conditions of ionic strength does the enzyme disaggregate to active species smaller than 6.4 to 6.5 S. Sodium dodecyl sulfate-polyacrylamide gel analysis of the purified enzyme shows maximal activity with gapped duplex DNA and has an absolute requirement for 3'-hydroxyl termini. It utilizes initiated polydeoxynucleotide templates poorly and initiated polyribonucleotide templates not at all. Although the polymerase is inhibited by PPi, it has only minimal ability to promote PPi exchange (0.8% of the polymerase activity). The purified enzyme is free of endonuclease and exonuclease activities (<0.003% of the polymerase activity) and demonstrates no primer-template-dependent conversion of substrate dNTP to free dNMP during the polymerization reaction. Finally, DNA polymerase C does not excise mispaired primer termini from a synthetic homopolymer primer-template but can utilize such termini as initiation sites, although at a very slow rate.

In recent years it has been recognized that eukaryotic cells contain at least four classes of DNA polymerase activity that can be distinguished to varying degrees by their apparent subcellular distribution and by physical, enzymatic, and immunological properties. These generally accepted classes include a DNA polymerase species that is associated with mitochondria (1-4); a physically heterogeneous (6 to 12 S) high molecular weight activity, usually derived from the cytoplasmic fraction of aqueously extracted cells (5-16), which we have designated KB cell DNA polymerase C (17, 18), a homogeneously sedimenting (3 to 3.5 S) low molecular weight polymerase, obtained from purified nuclei (7, 9-11, 13, 14, 19-22), which we have designated KB cell DNA polymerase N1 (17, 18, 23); and an activity that has been identified both in nuclear and cytoplasmic fractions and that shows a marked predilection for the replication of oligodeoxynucleotide-primed homopolyribonucleotide templates, particularly of the structure oligo(dT)-poly(A) (R-DNA polymerase) (24-29).

Although the precise roles played by these enzymes in vivo DNA replication remain to be established, particular interest has focused on the 6 to 12 S "cytoplasmic" polymerase for several reasons. First, this species constitutes the majority of the total DNA polymerase activity that is ordinarily recovered from aqueously prepared crude extracts of growing cells. Second, the activity of this species, in contrast to the 3 to 3.5 S nuclear polymerase, is responsive to cell proliferation, increasing significantly when quiescent cells are stimulated to divide (30, 31) and falling sharply as cell division ceases (32, 33). Third, a number of reports have described in purified nuclear fractions a high molecular weight DNA polymerase activity (9, 17, 18) whose presence and quantity appear to be closely correlated with active cell multiplication (13, 21, 34). Our physical studies reported here and recently published immunological evidence (35) suggest that this large nuclear polymerase and the "cytoplasmic" enzyme are very similar, if not identical. Fourth, although the "cytoplasmic" enzyme was the first eukaryotic DNA polymerase to be identified (5), it has thus far proved particularly difficult to purify. Thus the structure of the enzyme, the significance of the physical heterogeneity that it exhibits in crude or partially purified preparations (16, 35-37), its proposed association with a cytoplasmic smooth membrane fraction (10, 21), and its putative relationship to other eukaryotic DNA polymerases, particularly to the 3.5 S nuclear enzyme (38-41), remain matters of current controversy.

In previous publications we have described the isolation and partial characterization of three DNA polymerases from freshly harvested KB cells (17, 18) and have presented a detailed analysis of the structure and properties of a nearly homogeneous preparation of DNA polymerase N1 (23). This report complements the
latter and addresses the structure and properties of DNA polymerase C. The data to be presented will substantially clarify several of the controversial issues outlined above.

**EXPERIMENTAL PROCEDURE**

**Materials**

Unlabeled deoxyribonucleotides were purchased from P-L Biochemicals; H- and P-deoxyATP from Schwars/Mann and New England Nuclear, and α-[^32P]dTMP and α-[^32P]dATP were prepared by the exchange of ^32P; with H[^32P]dATP, utilizing Bacteriophila coli DNA polymerase I as described before (23) and further purified as described under "Methods." Both H- and ^32P-deoxyribonucleotides were purified by chromatography on Whatman No. 1 or 3MM paper with the solvent system, isobutyreric acid concentrated NH4OH/H2O (66:33). ^32P was purified by chromatography on Dowex 1 (42).

Standard protein markers, conalbumin, ovalbumin, and cytochrome c were purchased from Calbiochem; γ-globulin (human) and chymotrypsinogen (beef pancreas) from Mann Research, Inc; and 7 S γ-globulin (bovine) from Sigma. Acrylamide, bisacrylamide, bis(methylacrylamide), N,N,N',N'-tetramethylethylenediamine, and triethanolamine were obtained from Eastman Kodak. The acrylamide and bis(methylacrylamide) were routinely recrystallized for 5'-3'-exonuclease activity measures the generation of acid-soluble dTMP from [α-[^32P]d(pT)1-d(pT)G-dd(pT)1 with a 5'-3' exonuclease activity measures the generation of acid-soluble [α[^32P]dTMP from [α[^32P]d(pT)1-d(pT)100-d(pT)1]. The data to be presented will substantially clarify several of the controversial issues outlined above.

**Table I**

| Fraction or step | Volume (ml) | Protein (mg) | Activity (units/mg) | Specific activity (units/mg) | Yield (%) |
|------------------|-------------|-------------|--------------------|-----------------------------|-----------|
| I. Crude extract | 2,500       | 22,500      | 73,500             | 3.3                         | 100       |
| II. Acid precipitation | 200 | 9,100      | 68,000             | 7.5                         | 92.0      |
| III. Ultracentrifugation | 150 | 6,600      | 61,800             | 9.4                         | 84        |
| IV. DEAE | 345 | 260        | 48,000             | 185                         | 65        |
| V. Phosphocellulose I | 70 | 15.7       | 29,750             | 1,895                       | 40        |
| VI. Phosphocellulose II | 77 | 2.9        | 21,261             | 7,281                       | 29        |

**Methods**

**Growth of KB Cells**—The KB cells used in this study were grown, harvested, and fractionated for enzyme preparation as before (17, 18).

**Assay of DNA Polymerase C**—The standard assay with activated DNA primer-template has been described (17, 18). The standard assay with homopolymer primer-templates contained the following in a total volume of 0.10 ml: Tris-HCl, pH 7.6, 50 mM; MnCl2, 0.25 mM; dithiothreitol, 1 mM; bovine serum albumin, 100 μg/ml; Tris-HCl, 0.0270 sodium dodecyl sulfate and then lyophilized to dryness. The dry pellet was resuspended in 1/50 of the original sample volume of 10% glycerol in H2O and then boiled for 5 min in a 100° water bath. For NaCl treatment, enzyme fractions were first dialyzed against the desired molarity of NaCl for at least 2 hours and then dialyzed extensively against several changes of the Tris-sodium sulfate buffer and processed as described above. (c) Reduction and alkylation of enzyme fractions with β-mercaptoethanol and iodoacetamide were performed by a modification of a standard procedure (45). Protein (10 to 50 mg/ml) was equilibrated with H2O for 2 to 3 hours. For each 1% of protein, dithiothreitol was added to 7 μM, and the solution was incubated for 1 hour at 37°. For each 1% of protein, iodoacetamide was added to 20 μM. The protein solution was incubated with the alkylating agent for 1 hour at 0° and then prepared for sodium dodecyl sulfate treatment as above.

**Assay of Exonuclease Activities**—The assay for 3′-5′-exonuclease activity was carried out exactly as before (23). The assay for 5′-3′-exonuclease activity measures the generation of acid-soluble [α[^32P]dTMP from [α[^32P]d(pT)1-d(pT)100-d(pT)]. The data to be presented will substantially clarify several of the controversial issues outlined above.
inhibition of enzyme activity.

DNA polymerase incubation leads to an approximately 50 to 60% inhibition, since use of the doubly labeled dATP in a standard assay measures the incorporation of \[\text{[32P]}\text{dTMP} \text{next to [3H]}\text{dCMP} \text{with the} \ \text{primer-template, (dA)}^n \text{.(dT)}^m \text{-[3H]}\text{(dC)}_o \text{n.}\]

The assay was performed exactly as before (23). To measure PPi exchange, which is incorporated into the polymer chain, the reaction mixture was assayed for \[^{32}\text{P}]\text{Pi} \text{release (23).}\]

Generation of dNMP from dNTP during Polymerization—The assay was carried out exactly as before (23).

Other Methods—All other procedures used in these studies have been previously described (17, 18, 23). Protein was determined in purified enzyme fractions, VI and VII, protein was also estimated from Coomassie brilliant blue (R-250) stain intensity in sodium dodecyl sulfate polyacrylamide gels. Methods used for electron microscopy examination of subcellular fractions are described in the legend for Fig. 1. We are indebted to our colleague, Dr. K. Bensch, for preparing the electron micrographs and aiding us in their interpretation.

RESULTS

Purification of DNA Polymerase C

DNA polymerase C was purified from the cytoplasmic crude extract through the DEAE step (Fraction IV) as previously described (17, 18) (Table I). The active fractions were pooled and concentrated by dialysis against 10 volumes of 0.15 M potassium phosphate buffer, pH 7.2, containing 30% sucrose and 20% ethylene glycol. All buffers used in the subsequent phosphocellulose column steps contained 20% ethylene glycol and 1 mM each of EDTA and \(\beta\) mercaptoethanol.

Phosphocellulose Column Chromatography

The concentrated enzyme fractions were loaded on a phosphocellulose column (9.5 \(\times\) 7.5 cm), and the column was washed with 0.15 M KPi, pH 7.2, until all unabsorbed protein was removed. Enzyme activity was eluted in a single step with 0.22 M KPi. The peak fractions were pooled and concentrated by dialysis against solid sucrose at 4°C (Fraction V).

Second Phosphocellulose Column Chromatography

The concentrated enzyme fractions were adjusted to 0.15 M KPi, pH 7.2, and loaded on a phosphocellulose column (2.5 \(\times\) 7.5 cm). The column was washed with 3 bed volumes of the same buffer and then developed with a 50-ml linear gradient of 0.15 M to 0.31 M KPi, pH 7.2. The peak fractions of enzyme activity were pooled, concentrated by dialysis against solid sucrose, and stored over liquid nitrogen (Fraction VI). After 6 months of storage, about 40% of Fraction VI activity was lost, but longer storage for over 1 year did not result in any further loss of activity. In each of the three steps of column chromatography in this purification protocol only a single major peak of DNA polymerase activity is detected by the standard assay with activated DNA primer-template. Although the practice of pooling peak tubes at each step might lead to the discarding of minor polymerase species, we have no evidence to suggest the presence of additional significant species of D-DNA polymerase activity in our Fraction III preparations.

Sephadex G-200 Gel Filtration

When Fraction VI was filtered on a column (1.5 \(\times\) 20 cm) of Sephadex G 200 that had been equilibrated with 0.01 M Tris HCl, pH 7.9, 1 mM each of EDTA and \(\beta\) mercaptoethanol, and 10% glycerol, about 70% of the loaded enzyme activity was recovered in a single peak that eluted close to the void volume (\(V_v/V_o = 1.06\) (Fraction VII). Of note was the fact that Fraction VII contained only 5 to 10% of the total Coomassie blue staining material that was present in Fraction VI, which might indicate a specific activity of Fraction VII enzyme of \(>100,000\). However, since Coomassie blue is not a specific stain for protein on sodium dodecyl sulfate polyacrylamide gels. Methods used for electron microscopic examination of subcellular fractions are described in the legend for Fig. 1. We are indebted to our colleague, Dr. K. Bensch, for preparing the electron micrographs and aiding us in their interpretation.

Distribution of Polymerase C during Subcellular Fractionation

Bari et al. (10, 21) have reported that the cytoplasmic DNA polymerase from rat liver is associated with a subcellular fraction that is rich in smooth endoplasmic reticulum. We attempted to confirm this observation by fractionating freshly harvested KB cells according to the protocol of Baril et al. (10). Although the KB cell cytoplasmic DNA polymerase activity was indeed associated with a pellet fraction (corresponding to fraction P4 of Baril et al. (10)) containing smooth endoplasmic reticulum, the polymerase activity could be clearly separated from the membrane-rich fraction by subsequent sedimentation through a discontinuous sucrose gradient. As shown in Fig. 1, DNA polymerase C activity is found predominantly in a region of the gradient that contains no recognizable structures other than occasional dense material that resembles glycogen.

Structure of DNA Polymerase C

Analysis by Sucrose Gradient Sedimentation—The sedimentation behavior of DNA polymerase C at different stages of purification was determined by sucrose gradient sedimentation. As shown in Fig. 1, DNA polymerase C activity is found predominantly in a region of the gradient that contains no recognizable structures other than occasional dense material that resembles glycogen.
FIG. 1. Distribution of cytoplasmic DNA polymerase activity during subcellular fractionation of KB cells. The figure shows the recovery of DNA polymerase C activity from a discontinuous sucrose gradient (A) and representative fields of Gradient Fractions 2 (B), 3 (C), and 4 (D) as observed by electron microscopy. Freshly harvested KB cells were suspended in 0.025 M Hepes buffer, pH 7.5, 1 mM EDTA, 2 mM MgCl₂, and 1 mM β-mercaptoethanol and were broken by Dounce homogenization (17). The resulting crude extract was adjusted to 0.025 M Hepes, pH 7.5, 3 mM MgCl₂, 0.33 mM EDTA, 1 mM β-mercaptoethanol, 20 mM KCl, and 0.2 M sucrose. Nuclei and mitochondria were removed by centrifugation at 10,000 × g for 15 min, and microsomes were pelleted by centrifugation at 78,000 × g for 90 min. The supernatant was then centrifuged at 78,000 × g for 16 hours to obtain a pellet fraction that is essentially equivalent to the P₄ fraction of Barile et al. (10). 68% of the initial DNA polymerase activity in the crude extract was recovered in this step, 47% in the pellet and 21% in the supernatant. The pellet was carefully suspended in 0.025 M Hepes, pH 7.5, 5 mM MgCl₂, 25 mM KCl, 1 mM β-mercaptoethanol, and 0.25 M sucrose by homogenization with a loosely fitting pestle in a Dounce homogenizer. The suspension containing 2,074 units of polymerase activity was layered on a discontinuous sucrose gradient and centrifuged at 100,000 × g for 15 hours. The composition of the gradient, the fractions that were collected, and the total units of DNA polymerase activity (assayed under standard conditions with activated DNA primer-template) in each fraction are shown in A. Recovery of the enzyme activity from the gradient was 59%. Gradient Fractions 2, 3, and 4 were then individually centrifuged at 78,000 × g for 16 hours. A portion of each of the three pellets thus obtained was fixed in 2% phosphate-buffered glutaraldehyde (50), pH 6.7, postfixed in 1% OsO₄ (51, 52), dehydrated, and embedded in epoxy resin (Maraglas, Polysciences, Inc.; Rydal, Pa.). Ultrathin sections were double-stained with uranyl acetate and lead citrate (53) and examined with a Siemens Elmiskop 101 electron microscope (Siemens Corp. Iselin, N. J.) (magnification, × 66,000). Gradient Fraction 2 (B) contains predominantly smooth membrane material; Gradient Fraction 4 (D), free ribosomes with occasional membrane fragments. Most of the polymerase activity is in Gradient Fraction 3 (C), which contains few if any recognizable structures.
The recovery of loaded enzyme activity was always better than 90% (Table II).

The sedimentation of Fraction IV enzyme was examined after preparation either by the standard procedure (Table I) or by a modified protocol from which the step of acid precipitation (Step II) had been omitted. Sedimentation of the modified Fraction IV enzyme produced an activity profile (Fig. 2E) very similar to that observed with frozen crude extract, while sedimentation of the standard Fraction IV preparation (Fig. 2E) showed a substantial shift of the activity profile toward predominance of the 8 S component. This shift is not due to the acid precipitation step itself, however, since acid precipitation of freshly prepared Fraction I enzyme does not significantly alter the sedimentation profile illustrated in Fig. 2B. As observed with the crude extracts, centrifugation of Fraction IV enzyme in gradients containing 0.45 M NaCl resulted in quantitative conversion of the enzyme activity to the 6.4 S species (Table II). Finally, sedimentation of Fraction VI enzyme in gradients with or without NaCl (Fig. 2F) revealed only a single peak of enzyme activity at 6.5 S, although different preparations of Fraction VI occasionally showed some skewing of the activity profile toward higher S values in gradients lacking NaCl.

Analysis by Gel Filtration—The results of Sephadex G-200 gel filtration analysis of DNA polymerase C are presented in Fig. 3. At low ionic strength, Fraction II enzyme from the freshly prepared cytoplasmic extract sedimented as a relatively homogeneous single species with a peak at about 8 S and 10 S (Fig. 2A). In contrast, in the same gradients, the polymerase activity in frozen crude extracts exhibited the usual heterogeneous sedimentation profile with peaks at about 8 S and 10 S (Fig. 2A). Sedimentation analyses on linear 5 to 20% (w/w) sucrose gradients from the experiment in C were dialyzed against 0.01 M Tris-HCl, pH 7.9, concentrated by forced dialysis against Sephadex G-200 beads, and then recentrifuged as in A. E, standard Fraction IV enzyme (17, 18) (see text), no salt; O-O, modified Fraction IV enzyme (omission of acid precipitation step; see text), no salt. F, Fraction VI enzyme, no salt.

Estimation of the apparent Mr of Fraction VI enzyme by a standard plot (Fig. 3C) yielded values of about 253,000 (no salt) and 190,000 (0.45 M salt), respectively. These values are considerably larger than those predicted from sedimentation coefficients (Table II) and demonstrate the anomalous behavior of this enzyme on gel filtration that has been noted with crude enzyme fractions from calf and rat by others (15, 16, 36). To obtain a possibly more valid approximation of the size of the Fraction VI polymerase activity we estimated Stokes radius values from our gel filtration data (56, 57) (Fig. 3D) and used these, together with the S values (Table II) obtained under identical conditions of buffer composition and ionic strength, to compute Mr values of 170,000 (with salt) and 200,000 (without salt), respectively.

Analysis by Sodium Dodecyl Sulfate-Polyacrylamide Slab Gel Electrophoresis—The problem of severe protein aggregation that has frustrated previous efforts (17, 58) to display polymerase C protein in nondenaturing polyacrylamide gels persisted with the more highly purified enzyme fractions described here and was not overcome by boiling the preparations in sodium dodecyl sulfate. When Fraction VI was subjected to the several treatments described under “Methods” prior to electrophoresis in sodium dodecyl sulfate linear gradient gels, complex and variable
patterns of Coomassie blue staining material were obtained. However, in all preparations we consistently observed the presence of one or both of two discrete bands, which we have designated Bands A (Mr of 175,000) and B (Mr of 87,000), respectively.

Sodium dodecyl sulfate-polyacrylamide gel analysis of Fraction VII enzyme indicated that 90 to 95% of the total Coomassie blue stainable material in Fraction VI was removed by gel filtration, but there was persistence of protein Bands A and B. To determine whether these bands might be related to the polymerase C protein, two portions of Fraction VI enzyme were separately filtered through Sephadex G-200, and the elution profile of polymerase activity was determined. In one case, each fraction in the activity peak was individually reduced and alkylated and then subjected to sodium dodecyl sulfate gel electrophoresis. By this procedure, both Bands A and B were detected, and they represented >60% of the total Coomassie blue stainable material in the gel (Fig. 4A). The protein concentration of Band A plus Band B closely followed the profile of polymerase C activity (Fig. 4B). In the second case, each fraction across the peak of activity was separately treated with 2 mM Tris-HCl, pH 7.9, containing 1 mM each of p-mercaptoethanol and EDTA.

Some Comments Regarding KB Cell DNA Polymerase N2—We have previously (17, 18) reported the presence of a high molecular weight DNA polymerase activity in purified nuclear fractions from KB cells. This activity, DNA polymerase N2, could be clearly distinguished from DNA polymerase N1 and seemed also to differ in certain enzymatic properties from DNA polymerase C. However, polymerase N2 had not been highly purified, and thus its identification as a separate polymerase species remained uncertain. We have examined some of the physical properties of a preparation of polymerase N2 that had been purified through the step of DEAE-cellulose column chromatography (17, 18). When this fraction was centrifuged through sucrose gradients in the absence or presence of salt, sedimentation profiles identical with those illustrated in Fig. 2, A and C, were obtained. Under no condition of ionic strength up to 4 M NaCl have we observed conversion of polymerase N2 to active forms of a size resembling polymerase N1. Moreover, the polymerase N2 species were similar to polymerase C, and clearly different from polymerase N1 in terms of their extreme sensitivity to 0.2 M KCl and 30 mM p-hydroxymercuribenzoate (17, 18). Spadari et al. (35) have recently described a very close degree of antigenic relatedness between the HeLa cell DNA polymerases that correspond to KB cell polymerases C and N2. Thus the preponderance of the evidence now available suggests that polymerase N2 is very similar, if not identical, to DNA polymerase C.

**Enzymatic Properties of DNA Polymerase C**

**pH Optimum**—The pH optimum of polymerase C with activated DNA primer template is relatively broad (Fig. 5A) but shows a peak at pH 9.25 with Mg2+ as divalent action. In contrast, with (dT)20-poly(dA) as primer-template and Mn2+ as divalent cation, the pH profile of the enzyme peaks sharply at pH 7.6.
Recovery of loaded Fraction II activity were performed under standard conditions with activated DNA primer-template, Mg²⁺ is the preferred cation at an optimum concentration of 10 mM (Fig. 5C). With the primer-template (dT)₁₄-poly(dA), Mn²⁺ is most effective and there is a very sharp concentration optimum at 0.2 to 0.3 mM. No polymerase activity is detected with the homopolymer primer-template when Mg²⁺ is the divalent cation.

**Primer-Template Utilization**—A detailed description of the primer-template requirements of polymerase C has previously been reported (17, 18). Of note here is the fact that under optimum incubation conditions, the activity of this enzyme with oligod(T)-initiated poly(dA) template is only about 5 to 10% of that observed with activated DNA. Although polymerase C can use oligoribonucleotide initiators (17, 18), it is completely unable to copy an oligodeoxynucleotide initiated polynucleotide template (17, 18). Thus, incubation of Fraction VI enzyme for 60 min under optimum conditions with activated DNA primer-template at 37°, with (dT)₁₄-poly(dA) at 35°, or with (dT)₁₄-poly(dA) at 35° or at 30° (16, 24), resulted in the incorporation of 511, 38, and <0.18 pmol of [³²P]dTMP, respectively. There appears to be no general concurrence that the “cytoplasmic” DNA polymerase from a variety of sources can utilize oligoribonucleotide initiators very well (12, 14, 16, 59-61) but cannot copy polynucleotide templates (12, 14, 16, 28, 29, 61).

**Kinetics of Polymerization**—Under standard conditions with activated DNA primer-template (Fig. 5D), dNMP incorporation is brisk and reasonably linear for 120 min but ceases soon thereafter. The termination of the reaction is due to enzyme instability, since the addition of fresh enzyme at 120 min (Fig. 5D) reproduces the initial kinetics of incorporation.

5'→3'-Exonuclease Activity—KB DNA polymerase C and *Escherichia coli* DNA polymerase I were incubated under standard KB homopolymer conditions with [³²P]d(pT)₄-d(pT)₄ as substrate, with Mn²⁺ as divalent cation, and in the presence or absence of dTTP. Under these conditions the *E. coli* polymerase generated acid-soluble [³²P]dTMP at a rate equal to 5% of its rate of polymerization with activated DNA. By contrast, 3'-5'-exonuclease activity could be excluded from polymerase C to a level of <10⁻⁴% of its polymerizing activity.

3'→5'-Exonuclease Activity—DNA polymerase C had no detectable 3'→5'-exonuclease activity (<0.002% of the polymerizing activity) when tested with a double-stranded DNA substrate that was very highly labeled at every 3'-terminus (23) (Table III). Under these same assay conditions, the 3'-5'-exonuclease activity of *E. coli* polymerase I was readily detected at a ratio of nuclelease activity to polymerase activity of 0.94%. When polymerase C was challenged with a 3'-terminally labeled single-stranded DNA substrate, or with a 3'-terminally mismatched homopolymer substrate, a trace level of exonuclease activity was observed that was close to the limit of resolution of the assay (Table III). In contrast, when the same mismatched homopolymer was exposed to *E. coli* polymerase I, complete hydrolysis of the mispaired dCMP residues to the theoretical limit predicted from the known degree of enzyme saturation occurred within 2.5 min. We believe that the trace of 3'→5'-exonuclease activity that is present in polymerase C Fraction VI cannot be considered a significant polymerase-associated activity and is most likely due to a minor contaminant.

*The abbreviations used are: dd(pT)₄, 2',3'-dideoxy TMP; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.*
Elution Volume (ml)

**FIG. 4.** Correlation of Protein Bands A and B with Fraction VII polymerase activity. Fraction VI enzyme, 298 units in each experiment, was filtered on a Sephadex G-200 column (1.5 X 20 cm; void volume, 13 ml) that had been equilibrated with 0.01 M Tris-HCl (pH 7.9), 1 mmol each of EDTA and β-mercaptoethanol, and 10% glycerol. Polymerase activity (C—C) was recovered in a single peak (V/Vo = 1.06) (only those fractions that contained enzyme activity are shown in B and D); recovery of loaded activity was 45% in B, 71% in D. A and B, each of the fractions in the activity peak was separately reduced and alkylated and then analyzed by sodium dodecyl sulfate gel electrophoresis. The gel lane corresponding to the peak fraction of enzyme activity in Panel B is shown in Panel A; Bands A and B comprise 62% of the total Coomassie blue staining material in the gel. The histogram in Panel B shows the concentration of protein in Band A (shaded bars) and Band B (open bars) in each of the enzyme fractions. The total polymerase concentration of Band A plus Band B in each fraction is represented by the closed circles. (The details of protein quantitation are described under “Methods”). C and D, each of the fractions of the activity peak was separately treated with 2 M NaCl and then analyzed by sodium dodecyl sulfate gel electrophoresis. The gel lane corresponding to the peak fraction of enzyme activity in Panel D is shown in Panel C; Band B comprises 64% of the total Coomassie blue staining material in the gel. The concentration of protein in Band B in each enzyme fraction is represented by the closed circles. The standard marker proteins are: 1, bovine γ-globulin; 2, conalbumin; 3, ovalbumin; 4, chymotrypsinogen; 6, cytochrome C.

**PPi Release—**During the polymerization reaction, DNA polymerase C cleaves PPi from substrate dNTP, and dNMP incorporation and PPi release are in good stoichiometric agreement. Thus, with the doubly labeled substrate [3H, β-32P]dATP and under standard incubation conditions with DNA primer-template, an amount of polymerase C that incorporated 35 pmol of [3H]dAMP from [3H]dATP led to the incorporation of 14 pmol of [3H]dAMP from the doubly labeled substrate, with release of 15.9 pmol of free PPi.

**PPi Exchange—**Unlike KB polymerase N1 (23), polymerase C carries out a detectable PPi exchange reaction, but at an extremely low level (0.8% of the polymerase activity). At 2 mM PPi, at which polymerase C activity is inhibited about 40%, 1357 pmol of dTMP were incorporated in 30 min and only 19 pmol of PPi were exchanged. As we have earlier pointed out (23), one cannot attempt to drive the PPi exchange reaction by raising PPi concentration above 2 mM under these incubation conditions because of the formation of insoluble complexes of PPi with the divalent cation.

**dNTP Utilization by DNA Polymerase C—**The primer-template-dependent conversion of substrate dNTP to free dNMP during the polymerization reaction (62, 63), or dNTP turnover activity (64), is an associated activity that was initially recognized with E. coli DNA polymerase I and has been studied in considerable detail with the DNA polymerase of bacteriophage T4. We have previously described (23) a prominent dNTP turnover activity in our nearly homogeneous preparations of KB polymerase N1. In contrast, with DNA polymerase C we have been unable to detect this activity under any conditions of polymerization that were tested (Table IV). When the incorrect substrate, dCTP, was tested with the primer-template (dT)poly(dA), there was neither incorporation nor turnover of dCTP. This result is identical with that we previously reported (23) with KB polymerase N1 and E. coli DNA polymerase I and differs from the prominent turnover of a mispaired dNTP that is exhibited by the DNA polymerase of bacteriophage T4 (63).

**Reaction of DNA Polymerase C with a 3'-Terminally Mispaired Primer-Template—**Incubation of DNA polymerase C with the 3'-terminally mismatched primer-template, (dA)12-(dT)12-[3H]- (dC)12, and [α-32P]dTTP resulted in kinetics of dTMP incorporation that were quantitatively similar to those obtained with the homologous matched primer-template. Although a trace of 3'→5'-exonuclease activity could be detected in polymerase C Fraction VI by use of this mismatched homopolymer substrate (Table III), the number of dCMP termini that were hydrolyzed represented only 0.002 to 0.004% of the total dCMP residues in the reaction mixture. Since only about 50% of the primer chains in this mismatched primer-template contained dCMP residues at their 3' termini (23), a nearest neighbor analysis was performed on the reaction product to determine whether polymerase C was capable of using the mispaired termini as functional initiation sites. The results in Table V demonstrate a slow but progressive incorporation of [32P]dTMP next to [3H]dCMP terminal residues throughout the course of the 2-hour incubation.
VI activity was tested under standard conditions (0-0) or at higher pH (7.2, 8.0) with activated DNA primer-template. The pH of the incubation mixtures was varied as shown. The labeled substrate was [aH]dTTP. B, the effect of KC1 on Fraction VI was tested under standard conditions with (dT)z-poly(dA) as primer-template (O---O). The pH of the incubation mixtures was varied as shown. The labeled substrate was [aH]dTTP. C, the effect of divalent cation on Fraction VI activity was tested under standard conditions with (dT)z-poly(dA) as primer-template (O---O). The pH of the incubation mixtures was varied as shown. The labeled substrate was [aH]dTTP. D, the effect of divalent cation on Fraction VI activity was tested under standard conditions with (dT)z-poly(dA) as primer-template (O---O). The pH of the incubation mixtures was varied as shown. The labeled substrate was [aH]dTTP.

Since the fraction of total dCMP residues that was at the 3'-terminal position was 0.7 (23), the data indicate that by 120 min of reaction, about 10% of the mismatched termini had been utilized for polymerization.

**DISCUSSION**

This paper presents a detailed study of the structure and enzymatic properties of DNA polymerase C (17, 18), the KB cell counterpart of the high molecular weight polymerase activity that has been identified as the principal DNA polymerase species in a wide range of eukaryotic sources that include yeast (65), sea urchin (34), tetrathymana (66), chick embryo (14, 61), and diverse mammalian cells and tissues (5-7, 9-13, 15-18, 37). Although the "cytoplasmic" DNA polymerase was the first eukaryotic DNA polymerase to be isolated (5, 6), its molecular structure has not yet been established. The heterodisperse sedimentation profile that crude polymerase preparations exhibit in low ionic strength density gradients has proved perplexing and has led to uncertainty as to whether the "cytoplasmic" polymerase might be comprised of more than a single enzyme species (16, 31, 36), as well as to claims that the large polymerase is an aggregate form of the 3.5 S nuclear polymerase (39-41).

The DNA polymerase activity in the freshly prepared crude cytoplasmic fraction of aqueously extracted KB cell sediments in low ionic strength sucrose density gradients as a single discrete species of 10.8 S and is not associated with smooth membranes (10, 21) or with any other recognizable subcellular structure. The 10.8 S activity is quantitatively converted to a 6.4 S protomer species in the presence of 0.45 M NaCl, and this conversion is at least partially reversible. The shape of the sedimentation profile varies to some extent with the degree of enzyme purification, and the most highly purified preparations, polymerase Fraction VI, sediments identically as a homogeneous 6.5 S activity in the presence or absence of salt. From combined sedimentation and gel filtration analyses (69) we estimate the molecular weight of Fraction VI enzyme activity to be between 170,000 and 200,000. This estimate of the size of the active polymerase C protomer is in good agreement with those reported for the comparable polymerase activity from human lymphocytes (11), rat liver (15), and chick embryo (14).

Although DNA polymerase C has not yet been purified to homogeneity, sodium dodecyl sulfate-polyacrylamide gel analyses of Fraction VII suggest that protein Bands A and B, of 175,000 and 87,000 daltons, respectively, may be major components of the enzyme, and that Band A may be a dimer of Band B. Although not conclusive, those findings permit the interpretation that the active protomer of polymerase C is a 175,000-dalton dimer that is composed of two enzymatically inactive 87,000-dalton monomers. We note that Smith et al. (67) have observed a major band of protein of 89,000 daltons in a partially purified (specific activity, 359) preparation of the "cytoplasmic" DNA polymerase from cultured human lymphoblastoid cells.

The sedimentation and gel filtration data indicate that polymerase C has a pronounced tendency to associate at low ionic strength with itself or with other unidentified constituents of similar size (at least as defined by S value), and that our purification scheme results in the removal or alteration of factors that are required for this interaction. Although such aggregation has been encountered in most studies of "cytoplasmic" DNA polymerase activities from whatever source, we do not yet understand the phenomenon, nor do we know whether it may be of biological significance or is merely adventitious. Our findings do strongly suggest, however, that conclusions regarding multiplicity of "cytoplasmic" polymerase species (16, 36), interconversions between polymerase species (39-41), and hypothetical schemes of polymerase regulation (37, 40) that are based largely on alterations or differences in sedimentation and gel filtration profiles should be received with caution.

It is important to emphasize that by no method of analysis of DNA polymerase C that we have employed, neither by sedimentation nor gel filtration at salt concentrations up to 2 M, nor by sodium dodecyl sulfate gel electrophoresis, have we ever observed active enzyme species of 3 to 3.5 S or protein bands of 43,000 to 45,000 daltons that might correspond to DNA polymerase N1 (23). We underscore this point because there are several reports in the literature (38-41) that suggest that these two
polymerase species are structurally related. Our data do not
tween these two enzymes. Moreover, as we have previously noted
(20). With the 3'-terminally mismatched homopolymer substrate (14,170 cpm/pmol of dCMP
residue) (23), 3 units of polymerase C were incubated at 35° under
standard homopolymer conditions. In the last column, 3’-5’-exo-
nuclease activity is expressed relative to polymerase activity assayed
under standard KB conditions with activated DNA. The abbreviations used are: DS, double-stranded; SS, single-stranded.

| Enzyme                | Substrate        | dNTP present | dNMP generated/hr | dNMP generation %
|-----------------------|------------------|--------------|-------------------|----------------
| KB polymerase C       | DS DNA           | None         | <0.06             | <0.002         |
|                       | SS DNA           | None         | 0.015             |               |
|                       | (dA)_++(dT)_++[H](dC)_[7] | dTP          | 0.02              | 0.33           |
| E. coli polymerase I  | DS DNA           | None         | 37.7              | 0.94           |
|                       | SS DNA           | None         | 66.7              |               |

| Primer-template       | dNTP             | Labeled dNTP | Incorporation     | Generation   |
|-----------------------|------------------|--------------|------------------|--------------|
| Exonuclease III-      | dATP             | [3H]dATP     | 31.3             | <0.09        |
| treated DNA           | dGTP             | [3H]dGTP     | 26.3             | <0.30        |
|                       | dCTP             | [3H]dCTP     | 35               | <0.10        |
|                       | dTMP             | [3H]dTMP     | <0.7             | <0.7         |

| Time of incubation    | 3’-dTMP incorporation | 3’-dCMP incorporation | 3’-dATP incorporation | 3’-dGTP incorporation |
|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| min                    | fmol                  | fmol                  | fmol                  | fmol                  |
| 0                      | 243                   | 0.7                   | 0.00                  | 0.00                  |
| 30                     | 167                   | 5.7                   | 0.034                 | 0.008                 |
| 60                     | 154                   | 8.6                   | 0.056                 | 0.012                 |
| 120                    | 238                   | 16.6                  | 0.070                 | 0.014                 |

polymerase species are structurally related. Our data do not support this premise but agree with two recent studies (35, 68)
that could demonstrate no immunological cross-reactivity be-
tween these two enzymes. Moreover, as we have previously noted
(23) and as we amplify in an accompanying manuscript (43a),
der under certain conditions, particularly at low ionic strengths, DNA
polymerase N1 itself forms large aggregates that can be dissoci-
ted to the 3.5 S monomer in the presence of salt. The polymerase
N1 aggregate retains the characteristic enzymatic properties of the
monomer and can thus be unambiguously distinguished from the
polymerase C protomer or aggregate forms by appropriate
tests (17). However, given the strong propensity of both of these
classes of DNA polymerase to participate in ionic strength-de-
pendent aggregation reactions, and considering that crude cell
tissue extracts are usually prepared in hypotonic buffers that
favor such interactions, it is apparent that attempts to discrimi-
nate these classes of activity in impure preparations on the basis
of size criteria can be erroneous.  

We had previously reported that DNA polymerase C used
exonuclease III-treated DNA primer-template about 48% as well
as activated DNA primer-template (17, 18). We have subsequently
found that the relative degree of utilization varies profoundly with
different preparations of the former, probably reflecting differences
in the length of the gaps that are introduced and consequently the
actual primer-template conformation that the polymerase en-
counters.

Dr. N. B. Hecht has just sent us a preprint of a paper in press in
Biochim. Biophys. Acta in which he has clarified his earlier ob-
servations regarding the interconvertibility of the large and small
DNA polymerases. He has now obtained evidence that the cyto-
plasmic fraction of murine cells contains two species of large DNA
polymerase, one of which can be converted by salt to a 3.5 S
form. Hecht thus concludes that the salt-dissociable species is an
aggregate form of the small polymerase, while the non-dissociable
7 to 10 S species represents the "cytoplasmic" DNA polymerase.

TABLE III
3'-5'-Exonuclease activity in KB DNA polymerase C

| Substrate | Present | Generated/hr | % |
|-----------|---------|--------------|---|
| DS DNA    | None    | <0.06        | <0.002 |
| SS DNA    | None    | 0.015        |   |
| (dA)_+(dT)_+[H](dC)_[7] | dTP | 0.02 | 0.33 |
| E. coli polymerase I | None | 37.7 | 0.94 |
| SS DNA    | None    | 66.7         |   |

TABLE IV
Utilization of dNTPs by KB DNA polymerase C

| Primer-template | dNTP | Labeled dNTP | Incorporation | Generation |
|-----------------|------|--------------|---------------|------------|
| Exonuclease III-| dATP | [3H]dATP     | 31.3          | <0.09      |
| treated DNA     | dGTP | [3H]dGTP     | 26.3          | <0.30      |
| (dA)_+(dT)_+[H](dC)_[7] | dCTP | [3H]dCTP | 35 | <0.10 |
|                 | dTMP | [3H]dTMP     | <0.7          | <0.7       |

TABLE V
Reaction of KB DNA polymerase C with mismatched primer-
template (dA)_+(dT)_+[H](dC)_[7]

A reaction mixture was set up under standard homopolymer
assay conditions as described under "Methods." The incubation
(0.3 ml) contained 4.5 units DNA polymerase C; [α-32P]dTTP,
100 μM (9,000 cpm/pmol); (dA)_+[(dT)_+[H](dC)_[7] (14,170 cpm/pmol dCMP residue), 3.5 μM as primer.
Samples (50 μl) were removed at the indicated times for assay
of incorporated [32P]dTMP and for nearest neighbor analysis of the
polymer product as described in Ref. 23. The entries in the first
column show the recovery of 3'-[3H]dCMP residues from 3'-
primer termini. The data in the second column indicate the extent
of incorporation of [32P]dTMP next to dCMP termini: The ratios
in the third column show the fraction of total 3'-dCMP residues
recovered that had been joined in phosphodiester linkage to
dTMP.

| Primer-template | dNTP | Labeled dNTP | Incorporation | Generation |
|-----------------|------|--------------|---------------|------------|
| Exonuclease III-| dATP | [3H]dATP     | 31.3          | <0.09      |
| treated DNA     | dGTP | [3H]dGTP     | 26.3          | <0.30      |
| (dA)_+(dT)_+[H](dC)_[7] | dCTP | [3H]dCTP | 35 | <0.10 |
|                 | dTMP | [3H]dTMP     | <0.7          | <0.7       |
In striking contrast to the apparent structural complexity of DNA polymerase C, the enzymatic capabilities of the highly purified Fraction VI protomer are remarkably simple, an assessment that is in good agreement with that reached by Chang and Bollum (8) from their study of the comparable calf thymus enzyme. The general conclusion is now emerging (8, 23) that the eukaryotic DNA polymerases generally lack most or all of the associated properties that are so characteristic of their counterparts in prokaryotes (65). From the data presented here and in our earlier report (17), we may summarize the catalytic properties of polymerase C as follows. (a) With an appropriate primer-template, which may be either gapped DNA or oligodeoxynucleotide- or oligoribonucleotide-initiated polydeoxynucleotide template, polymerase C will incorporate correctly paired dNTPs at a very slow rate that is just below the limit of detection of our earlier study of polymerase N1 (23). Thus it remains possible that the latter polymerase might in fact possess a P1 exchange capability of this very low magnitude. (c) Polymerase C is devoid of endonuclease and exonuclease activities. (d) The enzyme cannot perform the dNTP turnover reaction, a capability that is prominently displayed by our homogeneous preparations of DNA polymerase N1 (3). (e) Like polymerase N1, polymerase C not only fails to excise mispaired primer termini, but it can utilize such termini as functional initiation sites, albeit at only about 10% of the rate observed with the nuclear enzyme. This lack of stringency with regard to the conformation of the 3'-primer terminus is not a peculiarity of the DNA polymerases derived from malignant or transformed cells since the same behavior has been clearly established for the small nuclear polymerase from calf thymus (8, 69). It thus appears that for both of the two well-defined classes of D-DNA polymerases, replication fidelity can be accomplished only by means of appropriate dNTP selection. If either polymerase encounters or introduces a mispaired dNMP, it possesses no mechanism for error correction and, at least in the %in vitro% situation, will replicate through it.

In the last few years studies in E. coli have sharply defined the obligatory participation of a large number of purified proteins and as yet uncharacterized gene products in meaningful, in vitro DNA replication (68, see Chapter 7). The use of more sophisticated primer-templates than activated DNA or synthetic homopolymers with eukaryotic DNA polymerases, in an effort to identify other, non-polymerase components of a “replication complex,” is a clear direction for future research in this area. This approach is perhaps made even more compelling by virtue of the very limited catalytic repertoire of those highly purified eukaryotic polymerases that have been characterized to date.

Acknowledgements—We acknowledge the expert technical assistance of Mrs. M. Sedwick and Mrs. L. Weiner.

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