DNA Polymerases from Bakers’ Yeast*

LUCY M. S. CHANG
From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

Two DNA polymerases are present in extracts of commercial bakers’ yeast and wild type Saccharomyces cerevisiae grown aerobically to late log phase. Yeast DNA polymerase I and yeast DNA polymerase II can be separated by DEAE-cellulose, hydroxylapatite, and denatured DNA-cellulose chromatography from the postmitochondrial supernatants of yeast lysates. The yeast polymerases are both of high molecular weight (>100,000) but are clearly separate species by the lack of immunological cross-reactivity.

Analysis of associated enzyme activities and other reaction properties of yeast DNA polymerases provides additional evidence for distinguishing the two species. Enzyme I has no associated nuclease activity but does carry out pyrophosphate exchange and pyrophosphorylation reactions. Enzyme II catalyzes pyrophosphate exchange and pyrophosphorylation reactions, and has an associated 3’-exonuclease activity. Enzyme I does not degrade deoxynucleoside triphosphates and cannot utilize a mismatched template. Enzyme II does carry out a template-dependent deoxynucleoside triphosphate degradation reaction and can excise mismatched 3’-nucleotides from suitable template systems.

Earlier studies have shown that both Enzyme I and Enzyme II are inhibited by N-ethylmaleimide. The yeast enzymes are not identical to any known eukaryotic or prokaryotic DNA polymerases. In general, Enzyme I appears to be most similar to eukaryotic DNA polymerase α and Enzyme II exhibits properties of prokaryotic DNA polymerases II and III.

Multiple DNA polymerases are present in both prokaryotic (1, 2) and multicellular animal cells (3–5). It is a matter of theoretical and technical interest to analyze detailed characteristics of DNA polymerases in a variety of biological systems. Analysis of DNA polymerases in simple eukaryotes (5–10) and plants (11) showed the absence of DNA polymerase β activity in these systems. In the limited data available (where partial purification and characterization of the DNA polymerases had been carried out) the properties of the enzymes seemed to vary with the species examined (6–9, 11). The purpose of this study was to compare certain properties of highly purified yeast DNA polymerases with other well characterized DNA polymerases from bacterial and mammalian sources (1–4, 12, 13).

Yeast is of special interest because preliminary studies indicated that fungi did not show a typical eukaryotic pattern of DNA polymerase activity (6). Although the absence of DNA polymerase β in yeast (6, 7) suggests that yeast may not be a model system for studies on DNA replication and repair in more complex eukaryotic cells, the presence of a well characterized genetic system should allow analysis of biological effects through mutation. The first question concerns whether either of the yeast DNA polymerases resembles the mammalian (3, 4) or bacterial enzymes (1, 2). A notable major difference between the bacterial and mammalian enzymes is that all bacterial DNA polymerases have associated exonuclease activity (1, 2) while the mammalian L1 DNA polymerases do not exhibit this property (12, 13). Earlier studies by Wintersberger (6) and Helfman (14) showed that an exonuclease activity co-purified with yeast DNA polymerase II. The results from this study confirm that yeast DNA polymerase I has no associated nuclease activity while yeast DNA polymerase II has an associated exonuclease activity. The exonuclease associated with yeast DNA polymerase II is a 3’-exonuclease and is capable of removing replication errors (1). Other properties of the yeast enzymes are also described.

MATERIALS AND METHODS

Yeast Strains

DNA polymerase activities were analyzed in the wild type Saccharomyces cerevisiae, strain D 278-10B (PET [p']) (15). The cells were grown aerobically to late log phase (about 5 g wet weight per liter) in a medium containing 1% yeast extract (Difco), 2% peptone (Difco), 2% dextrose, and a few drops of a silicone antifoam emulsion. After the cells were harvested and washed, a 90% suspension in 8.5% glycerol, 10 mM Tris/Cl at pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.6 mM phenylmethylsulfonyl fluoride, and 1% dimethylsulfoxide was prepared. The suspension was frozen by allowing drops to fall into liquid N₂. The N₂ was then poured off and the frozen pellets were stored at 70°C. Commercially grown bakers’ yeast (obtained from National Brands Inc., Baltimore, Md.) was used for the purification of the DNA polymerases.

Substrates

Deoxynucleoside triphosphates (dNTP)¹ and pancreatic DNase I activated calf thymus DNA were prepared as previously described (16). Polydeoxynucleotides and oligodeoxynucleotides were synthesized with calf thymus terminal deoxynucleotidyltransferase (17).

¹ The abbreviations used for nucleotides and polynucleotides are those of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) (1970) J. Biol. Chem. 245, 5171–5176. Abbreviations for homopolymers bearing a barred subscript refer to average chain length. A definite subscript number refers to exact chain length of oligonucleotides.
DNA polymerase reactions were routinely carried out in the presence of 0.05 M Tris/Cl buffer at pH 8.0, 2 mM 2-mercaptoethanol, 150 μg/ml of activated calf thymus DNA, 8 mM MgCl₂, 0.1 mM concentration of each of dCTP, dATP, and dGTP, and 0.1 mM [β-32P]dUTP at a specific activity of 50,000 to 150,000 cpm/ml. Assays carried out on fractions before any column purification also contained 2 mM ATP. After the phosphocellulose column all assays contained 100 μg/ml of bovine serum albumin. Synthetic template systems contained 0.1 mM template nucleotide, 0.01 mM RNA polymerase, 0.05 mM Tris/Cl at pH 8.0, 2 mM 2-mercaptoethanol, 0.2 mM complementary radioactive dTTP, 2.5 mM MgCl₂, and 100 μg/ml of bovine serum albumin. Products of the reaction were detected as acid-insoluble radioactivity as previously described (16). One unit of enzyme is defined as 1 nmol of total nucleotide polymerized per h.

Substrates for the nuclelease assays were used as described. Pyrophosphatase activities were carried out in the presence of 0.05 M Tris/Cl buffer at pH 7.4, 2 mM 2-mercaptoethanol, 150 μg/ml activated calf thymus DNA, 0.1 mM concentration of each of dTTP, dATP, dCTP, and dGTP, 100 μg/ml of bovine serum albumin. The amounts of radioactive substrates used in the nucleolytic assays were 0.12 mM and 0.18 mM 5'-labeled polymer nucleotides with micrococcal nuclease and 0.05 M Tris/Cl at pH 8.0, 2 mM 2-mercaptoethanol, and 10% glycerol. Pyrophosphorylases were carried out using the same reaction conditions in the absence of dNTPs. Analysis of the exchange and pyrophosphorylation products by adsorption onto charcoal was done as previously described (12).

Oligoribonucleotide triphosphate degradative reactions ("turnover") were carried out in 0.05 M Tris/Cl at pH 8.0, 8 mM MgCl₂, 2 mM 2-mercaptoethanol, 0.1 mM d[32P]ATP, 0.01 mM d[32P]dTTP, 2.5 mM MgCl₂, 0.2 mM (2′-3′)dATTP (4200 cpm/ml), and 100 μg/ml of bovine serum albumin. The products of the reactions were analyzed by paper chromatography as previously described (12).

**Protein Determination**

Protein concentrations were determined with the biuret reagent (20) for fractions obtained prior to phosphocellulose column chromatography, and by absorbance at 280 nm for subsequent fractions.

**Isoelectric Focusing**

Isoelectric focusing of the yeast DNA polymerases was carried out on a micro scale in an apparatus described by Godson (21). The focusing column consisted of a 10-ml 10 to 30% linear sucrose gradient containing protein to be analyzed, 10% ethylene glycol, and 0.1% ampholine with a pH range of 5.5 to 10 (LKB Produkter A. B., Stockholm). Focusing was carried out for 6 h at 800 V at 4°C. The sucrose gradient was fractionated into 0.28-ml fractions and collected in tubes containing 25 μl of solution of 0.75 M potassium phosphate at pH 7.5, 5 mg/ml of bovine serum albumin, and 5 mM 2-mercaptoethanol. DNA polymerase and nuclease activities in aliquots of these fractions were assayed as described above.

**Preparation of Antiserum**

Antiserum to yeast DNA polymerase I was obtained from a 4-kg male New Zealand rabbit. Prior to immunization, the rabbit was bled in order to obtain the control serum. Primary immunization was made at multiple subcutaneous sites (after 150 μg of a yeast DNA polymerase I preparation (0.7 ml, specific activity was 340,000 units/mg of protein) suspended in 0.7 ml of Freund's complete adjuvant). No inhibitory activity was found in serum obtained 2 weeks after the primary injection. A secondary immunization consisting of 40 μg of enzyme protein (650,000 units/mg) in Freund's complete adjuvant was administered 3 weeks after the primary injection. A weak anti- serum was obtained 2 weeks after the secondary immunization. A booster (identical to secondary immunization) was then administered and serum was collected 2 weeks later. This serum had a relatively high titer and was used for enzyme neutralization studies.

**Analysis of DNA Polymerases in Wild Type Yeast**

Frozen yeast cell pellets (equivalent to 60 g) were transferred to an Eaton Press and ruptured at 9000 p.s.i. The lysate was suspended in 120 ml of ice cold 0.05 M potassium phosphate buffer at pH 8.8, 1 mM EDTA, 0.12 M KCl, 1% dimethylsulfoxide, and 0.5 mM phenylmethylsulfonyl fluoride. All procedures were done at about 4°C. The lysate was clarified by centrifugation and 1/4 volume of 1 M Tris/Cl at pH 8.0 was added to the 24,000 g x l supernatant. The nucleic acids in the supernatant were removed by precipitation with 20% protamine sulfate. The protein in the protamine sulfate supernatant was precipitated with 60% saturation of (NH₄)₂SO₄. The precipitate was collected by centrifugation, redissolved in 0.05 M potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. The dialyzed fraction was further fractionated in a column (Whatman DE-32, 10 x 1874 cm) previously equilibrated with 0.05 M potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. The column was washed with the same buffer and eluted with 0.25 M KCl in the same buffer.

The active fractions from the phosphocellulose column were pooled, dialyzed against 25 mM potassium phosphate at pH 7.2 in 5 mM 2-mercaptoethanol, and loaded onto a DE11 cellulose column (Whatman 5 × 30 cm) previously equilibrated with the same buffer. The column was washed and then eluted with a 200-ml linear gradient of 0 to 0.5 M KCl in 25 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol.

**Purification of Yeast DNA Polymerase from Commercial Yeast**

**Step 1: Crude Extract—Post mitochondrial supernatant (3.6 liters)** was prepared according to Mason et al. (22) and was a generous gift from Dr. R. O. Poyton, University of Connecticut Health Center. The pH of the supernatant was readjusted to pH 7.6 by addition of 400 ml of 1 M Tris/Cl at pH 8.0. This solution is called Fraction I.

**Step 2: Protamine Sulfate Precipitation—One liter of 2.3% protamine sulfate solution was added to 4 liters of Fraction I with stirring. The extract was allowed to stand for 30 min and was then clarified by centrifugation for 30 min at 9000 rpm in the GS-3 rotor in the Sorvall centrifuge. The volume of the supernatant, Fraction II, was 3.9 liters.

**Step 3: Ammonium Sulfate Precipitation—Solid (NH₄)₂SO₄ was added to Fraction II to a final concentration of 60% saturation. The protein precipitate was collected by centrifugation and redissolved by addition of about 250 ml of 25 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 0.5 mM EDTA. The redissolved (NH₄)₂SO₄ fraction (800 ml) was dialyzed for 20 h against 30 liters of 25 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 0.5 mM EDTA. For 20 h against 30 liters of 50 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. Two peaks of activity were obtained from this column (cf. Fig. 1). Active fractions eluting from the column were combined, concentrated by ultrafiltration through 50000 molecular weight cut-off membranes, and dialyzed against 25 mM potassium phosphate, 5 mM 2-mercaptoethanol, and 10% glycerol. The final volume of concentrated Fraction IV was less than 100 ml.

**Step 5: DE11 Cellulose Column Chromatography—Dialyzed, concentrated Fraction IV was loaded onto a DE11 cellulose column (2.6 × 60 cm) previously equilibrated with 25 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. The column was washed with the same buffer and eluted with a 2.8-liter linear gradient of 0 to 0.5 M KCl in 25 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. The final volume of concentrated Fraction IV was less than 100 ml.
Yeast DNA Polymerases

Although commercial yeast cake provides a convenient source for the purification of DNA polymerases, it is essential to establish that the enzymes present are the same as those found in wild type Saccharomyces cerevisiae grown in defined media and under sterile conditions. Independent confirmation of the presence of a minor activity (DNA polymerase II) is particularly important.

The conditions used in comparing the crude extract from the wild type yeast cells and commercial yeast cells were similar with two minor exceptions. Phenylmethylsulfonyl fluoride, a protease inhibitor, was included in the extraction buffer used with wild type cells. A lower final concentration of protamine sulfate was used for wild type extracts since the extracts were more dilute.

The specific activity of DNA polymerase activity (7.7 units/mg of protein) in the crude extract of the wild type yeast cells was comparable to that of the crude extract of commercial yeast (5.5 to 10 units/mg in various preparation). Partial purification of the wild type extract appears to proceed much as for the extract of commercial yeast (see below). For example, 7,800 units out of 17,000 units were recovered after the phosphocellulose column. A net purification of about 25-fold was obtained prior to chromatography on DE11 column.

All activity found in the phosphocellulose fraction of the wild type extract was loaded onto the DE11 column and the DNA polymerase activities eluted from DE11 by a salt gradient are shown in Fig. 1. Two peaks of DNA polymerase activity are eluted from DE11 column of the wild type extract, comparable to results obtained with extracts of commercial yeast. Enzyme I, eluting at about 0.12 M KC1, was totally inhibited by the antiserum elicited by Enzyme I purified from the commercial yeast. Enzyme II (less than 10% of the total activity), eluting from the column at about 0.2 M KC1, was not inhibited by the antiserum against Enzyme I.

Purification of Yeast DNA Polymerase from Commercial Yeast Cells

The results of the more extensive purification procedure applied to extracts of commercial yeast are summarized in Table I. DNA polymerase I was purified 20,000- to 65,000-fold by this procedure, while DNA polymerase II was purified 8000- to 13,000-fold. Further research will be required for preparation of homogeneous enzyme in good yield. The removal of nucleic acids from crude extract by protamine sulfate precipitation is essential for the success of the purification. The A490 to A280 ratio in the crude extract, protamine sulfate supernatant, and the dialyzed (NH4)2SO4 fraction were 0.6, 1.1, and 1.6, respectively. When the (NH4)2SO4 fraction was fractionated on the phosphocellulose column, two poorly re-

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zyme protein. Due to the limited quantity of purified Enzyme II available for analysis, no distinct banding pattern could be prepared. The effects of this antiserum or Enzyme I and Enzyme II are shown in Fig. 2. The result shows the antiserum to which can be completely removed from Enzyme II on the hydroxylapatite column (Fig. 3A).

The gross reaction properties related to synthetic activity are not significantly different for the two yeast enzymes. Both enzymes have an absolute requirement of the presence of an initiated template and a divalent cation. When activated DNA is used as the template, maximum activity can be obtained only in the presence of all four dNTPs. In 0.05 M Tris/Cl buffer, pH optima were at pH 8.2 and pH 7.6, for Enzyme I and Enzyme II, respectively. The enzyme activities, however, were found to be 8 to 30% higher in 25 mM potassium phosphate at pH 8.0, and 50 mM Ammonium/Cl buffer at pH 7.8. For convenience, 50 mM Tris/Cl buffer at pH 8.0, was used for both enzymes in further characterization studies. The activities of the Enzyme I and Enzyme II in 50 mM Tris/Cl buffer were 91 and 79% of their corresponding activities at optimum.

When activated DNA was used as the template, the optimum magnesium ion concentration was 8 to 20 mM. Manganous ion at 0.8 to 1.2 mM catalyzed this reaction at about 10% of the magnesium catalyzed rates. When a synthetic template, such as d(pA)2.d(pT)8, was used as template, magnesium remained the preferred divalent cation. Enzyme I and Enzyme II have sharp magnesium optima at 3 mM and 2 mM, respectively. Manganous ion at 0.15 to 0.5 mM catalyzed the homopolymer replication reactions at about 10% of the optimum magnesium catalyzed rates.

TABLE I

| Purification of yeast DNA polymerases | Protein (units/mg) | Specific activity | Total enzyme activity (units/mg) |
|-------------------------------------|-------------------|-------------------|---------------------------------|
| I. Crude extract                    | 590,000           | 9.8               | 5.78 x 106                      |
| II. Protamine sulfate supernatant   | 298,000           | 20.0              | 4.16 x 106                      |
| III. Diaalyzed (NH4)2SO4            | 72,400            | 47.8              | 3.46 x 106                      |
| IV. Phosphocellulose                | 2,500             | 648               | 1.62 x 106                      |
| Enzyme I                            |                   |                   |                                 |
| Va. DE11 cellulose                  | 162               | 5,480             | 0.99 x 106                      |
| Vla. Sephadex G-200                 | 41                | 13,040            | 0.53 x 106                      |
| VIIa. Hydroxylapatite               | 5.6               | 55,300            | 0.31 x 106                      |
| VIIIa. Denatured DNA-cellulose      | 0.68              | 340,000           | 0.23 x 106                      |
| Enzyme II                           |                   |                   |                                 |
| Vb. DE11 cellulose                  | 0.7               | 1.4/0             | 100 x 10^6                      |
| Vlb. Sephadex G-200                 | 8.5               | 6,470             | 55 x 10^3                       |
| Vlb. Hydroxylapatite                | 0.63              | 29,900            | 19 x 10^3                       |
| VIIIb. Denatured DNA-cellulose      | 0.06              | 119,000           | 7 x 10^4                        |

In order to examine the possibility that the two yeast enzymes are related, antiserum to yeast DNA polymerase I was prepared. The effects of this antiserum or Enzyme I and Enzyme II are shown in Fig. 2. The result shows the antiserum to be completely specific for Enzyme I. No inhibition of Enzyme II activity was observed. This lack of cross-reactivity is fairly good evidence for the presence of two distinct enzymes in yeast.

Properties of Yeast DNA Polymerases

The antiserum was assayed as described under "Materials and Methods." Results were calculated as percentage of activity remaining in antiserum-treated sample compared to control serum-treated sample at comparable serum dilution.

A rigorous study of the purity of the enzyme preparations obtained have not been completed. Attempts to analyze native enzymes by electrophoresis or isoelectric focusing on acrylamide gels were unsuccessful because of diffuse banding patterns. Gel electrophoresis in the presence of sodium dodecyl sulfate of the best preparation of Enzyme I (specific activity was 650,000 units/mg of protein) showed two bands with molecular weight around 70,000. These two bands contained about 50% of the protein on gel. Since the molecular weight of both yeast DNA polymerases was estimated to be around 150,000 on Sephadex G-200 and sucrose gradient centrifugation, it is possible that these two bands on sodium dodecyl sulfate gels represent peptides of the enzyme protein. If this reasoning is correct, then the best preparation of Enzyme I we were able to obtain contained only about 50% enzyme protein, and the preparation reported in this communication contained less than 25% enzyme protein. Due to the limited quantity of purified Enzyme II available for analysis, no distinct banding pattern could be identified when Enzyme II preparations were run on sodium dodecyl sulfate gels.

Immunological Cross-reactivity of Yeast DNA Polymerases

In order to examine the possibility that the two yeast enzymes are related, antiserum to yeast DNA polymerase I was prepared. The effects of this antiserum or Enzyme I and Enzyme II are shown in Fig. 2. The result shows the antiserum to be completely specific for Enzyme I. No inhibition of Enzyme II activity was observed. This lack of cross-reactivity is fairly good evidence for the presence of two distinct enzymes in yeast.
The presence of salt stimulated the activity of both enzymes when activated DNA was used as template. Ammonium sulfate at 60 mM stimulated Enzyme I activity 90%. Enzyme II activity was stimulated 130% at 30 mM. Sodium chloride at 30 mM stimulated both enzyme activities by 50%. Potassium chloride at 60 mM stimulated Enzyme I and Enzyme II activities 90 and 110%, respectively. The concentrations for various salts presented are those giving maximum stimulation with activated DNA. When homopolymers were used as templates, both enzyme activities were inhibited by increased ionic strength. For example, at 30 mM KCl, Enzyme I and Enzyme II activities on initiated poly(dA) were inhibited by 35 and 60%, respectively.

A comparison of the affinities of the two yeast DNA polymerases for various substrates (Table II) shows that the $K_m$ values of the two enzymes for various substrates in polymerization reactions do not differ greatly. The affinity of Enzyme I for the template was found to be somewhat greater than Enzyme II. The $K_m$ values of the two enzymes for the four dNTPs were found to be about the same. Among the dNTPs, the enzymes seem to have slightly greater affinity for the purine nucleoside triphosphates than for the pyrimidines.

Template specificities of the yeast DNA polymerases are shown in Table III. The amount of each enzyme used in the reactions included in this comparison was an equal amount of enzyme activity as measured on d(pA)$_{10}$,d(pT)$_{10}$. As shown in Table III, the rate of polymerization on activated DNA was comparable to the poly(dA) replication rate for Enzyme I, but the rate on activated DNA was 3-fold lower than the rate of polymerization on d(pA)$_{10}$ for Enzyme II. Neither enzyme utilizes native or heat-denatured DNA effectively as a template. Enzyme I catalyzes polymerization on poly(dA)-dT template whereas Enzyme II was relatively inactive under identical conditions. Neither enzyme can catalyze the replication of oligo-thymidylyl-tiated poly(rA) in the presence of magnesium ion. When manganese ion was used, however, the rates of replication of poly(dA) and poly(rA) were found to be almost identical for Enzyme I. Oligo-thymidylylated was found to be an effective initiator for poly(dT) replication in the presence of either magnesium or manganese ion for Enzyme I. Enzyme II can also utilize oligo-thymidylylated-initiated poly(dT) template, although at much lower rate. Oligo-adenylate-initiated poly(dA) was found to be a poor template system for Enzyme I and is ineffective with Enzyme II.

** Associated Enzyme Activities in Yeast DNA Polymerases

** Associated Nuclease Activity — During the purification of the DNA polymerases from yeast, high levels of both 3’- and 5’-exonucleases were found in both Enzyme I and Enzyme II fractions through the Sephadex G-200 column step. All detectable nuclease activity was removed from Enzyme I by hydroxylapatite chromatography. Most of the nuclease activities eluted from hydroxylapatite column at potassium phosphate concentrations greater than 0.2 M. As previously noted, Fraction VII containing Enzyme II still contained Enzyme I after the Sephadex G-200 column. When Fraction VII was chromatographed on hydroxylapatite, Enzyme II was well separated from Enzyme I but coincided with a 3’-exonuclease activity (Fig. 3A). The ratio of DNA polymerase and 3’-exonuclease was found to be about 2.5. When the nuclease assays were carried out with d(pA)$_{10}$,$d(pA)$_{10}$,$d(pT)$_{10}$, the rates of hydrolysis of nucleotides from the chain containing radioactivity were about half of the rates of hydrolysis of the single-stranded substrate. Assuming the rate of hydrolysis of nonradioactive d(pT)$_{10}$ is the same as the radioactive d(pA)$_{10}$, no specificity on single- or double-stranded substrates can be demonstrated. No hydrolysis was observed when single- or double-stranded 5’-end-labeled substrates were used. The 3’-exonuclease co-fractionated with DNA polymerase II activity on the denatured DNA-cellulose column (Fig. 3B) and on isoelectric focusing (Fig. 3C). The ratio of polymerase to nuclease for Enzyme II remained at about 2.5.

The possibility that the DNA polymerase and nuclease activities in Enzyme II are on the same protein is further substantiated by heat inactivation studies. Fig. 4 shows that Enzyme I was inactivated at 48° faster than Enzyme II. The DNA polymerase and 3’-exonuclease activities of Enzyme II have identical heat inactivation kinetics.

The 3’-exonuclease associated with the prokaryotic DNA polymerases has been shown to exhibit a “proofreading” function in the enzymatic replication of DNA (1, 2, 25). The mammalian DNA polymerases do not have any associated nuclease and therefore are not able to excise replication errors (12, 13). Yeast cells seem to contain both types of DNA polymerase. It was a further interest to examine whether the yeast DNA polymerases do not have any associated nuclease activity, cannot excise a mismatched template but contain a mismatched 3’-terminal nucleotide.

**Deoxyribonucleotide Triphosphate Degradation — The presence of salt stimulated the activity of both enzymes when activated DNA was used as template. Ammonium sulfate at 60 mM stimulated Enzyme I activity 90%. Enzyme II activity was stimulated 130% at 30 mM. Sodium chloride at 30 mM stimulated both enzyme activities by 50%. Potassium chloride at 60 mM stimulated Enzyme I and Enzyme II activities 90 and 110%, respectively. The concentrations for various salts presented are those giving maximum stimulation with activated DNA. When homopolymers were used as templates, both enzyme activities were inhibited by increased ionic strength. For example, at 30 mM KCl, Enzyme I and Enzyme II activities on initiated poly(dA) were inhibited by 35 and 60%, respectively.

A comparison of the affinities of the two yeast DNA polymerases for various substrates (Table II) shows that the $K_m$ values of the two enzymes for various substrates in polymerization reactions do not differ greatly. The affinity of Enzyme I for the template was found to be somewhat greater than Enzyme II. The $K_m$ values of the two enzymes for the four dNTPs were found to be about the same. Among the dNTPs, the enzymes seem to have slightly greater affinity for the purine nucleoside triphosphates than for the pyrimidines.

Template specificities of the yeast DNA polymerases are shown in Table III. The amount of each enzyme used in the reactions included in this comparison was an equal amount of enzyme activity as measured on d(pA)$_{10}$,d(pT)$_{10}$. As shown in Table III, the rate of polymerization on activated DNA was comparable to the poly(dA) replication rate for Enzyme I, but the rate on activated DNA was 3-fold lower than the rate of polymerization on d(pA)$_{10}$ for Enzyme II. Neither enzyme utilizes native or heat-denatured DNA effectively as a template. Enzyme I catalyzes polymerization on poly(dA)-dT template whereas Enzyme II was relatively inactive under identical conditions. Neither enzyme can catalyze the replication of oligo-thymidylyl-tiated poly(rA) in the presence of magnesium ion. When manganese ion was used, however, the rates of replication of poly(dA) and poly(rA) were found to be almost identical for Enzyme I. Oligo-thymidylylated was found to be an effective initiator for poly(dT) replication in the presence of either magnesium or manganese ion for Enzyme I. Enzyme II can also utilize oligo-thymidylylated-initiated poly(dT) template, although at much lower rate. Oligo-adenylate-initiated poly(dA) was found to be a poor template system for Enzyme I and is ineffective with Enzyme II.

** Associated Enzyme Activities in Yeast DNA Polymerases

** Associated Nuclease Activity — During the purification of the DNA polymerases from yeast, high levels of both 3’- and 5’-exonucleases were found in both Enzyme I and Enzyme II fractions through the Sephadex G-200 column step. All detectable nuclease activity was removed from Enzyme I by hydroxylapatite chromatography. Most of the nuclease activities eluted from hydroxylapatite column at potassium phosphate concentrations greater than 0.2 M. As previously noted, Fraction VII containing Enzyme II still contained Enzyme I after the Sephadex G-200 column. When Fraction VII was chromatographed on hydroxylapatite, Enzyme II was well separated from Enzyme I but coincided with a 3’-exonuclease activity (Fig. 3A). The ratio of DNA polymerase and 3’-exonuclease was found to be about 2.5. When the nuclease assays were carried out with d(pA)$_{10}$,d(pA)$_{10}$,d(pT)$_{10}$, the rates of hydrolysis of nucleotides from the chain containing radioactivity were about half of the rates of hydrolysis of the single-stranded substrate. Assuming the rate of hydrolysis of nonradioactive d(pT)$_{10}$ is the same as the radioactive d(pA)$_{10}$, no specificity on single- or double-stranded substrates can be demonstrated. No hydrolysis was observed when single- or double-stranded 5’-end-labeled substrates were used. The 3’-exonuclease co-fractionated with DNA polymerase II activity on the denatured DNA-cellulose column (Fig. 3B) and on isoelectric focusing (Fig. 3C). The ratio of polymerase to nuclease for Enzyme II remained at about 2.5.
Fig. 3. Relationship of DNA polymerase and 3'-exonuclease functions in yeast DNA polymerases. Panel A, 4.3 A_{280} units of a fraction equivalent to Fraction VIb were analyzed on a hydroxylapatite column (1 x 3 cm). Total enzyme activity loaded onto the column was 21,000 units. The column was eluted with a 70-ml linear gradient of 0.05 to 0.2 M potassium phosphate at pH 7.2 in 5 mM 2-mercaptoethanol and 10% glycerol. Panel B, 0.4 A_{280} unit of a fraction equivalent to Fraction VIIb was analyzed on a denatured DNA-cellulose column (0.9 x 2 cm). Total enzyme activity loaded onto the column was 5,500 units. The column was eluted with a 60-ml linear KCl gradient (0 M to 0.2 M) in 50 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. Panel C, 190 units of a fraction equivalent to Fraction VIIIb were analyzed on an isoelectric focusing column as described under "Materials and Methods." The enzyme assays were carried out as described under "Materials and Methods" using activated calf thymus DNA as template for DNA polymerase assays and d(pA)_{254}[8-^3]Cld(pA)_{254} as substrate for 3'-exonuclease assays. The recovery of enzyme activities for each of the methods of analysis was more than 80%.

Fig. 4. Heat inactivation of yeast DNA polymerases. Yeast DNA polymerases, diluted in a buffer containing 5 mM Tris/Cl at pH 8.0, 5 mM 2-mercaptoethanol, and 50% glycerol, were distributed into 25.1 ml aliquots, each containing 10 units of enzyme. The aliquots were incubated at 48°C. Tubes were removed from the 48°C water bath at various times and plunged into an ice bath. Enzyme activity remaining was assayed as described under "Materials and Methods" using d(pA)_{254}, d(pT)_{254}, as template in DNA polymerase reactions and 0.1 mM [8-^3]Cld(pA)_{254} (8,000 cpm/nmol of deoxyadenylate) as substrate for the nuclease reactions.

of a 3'-exonuclease function on yeast Enzyme II suggests that this DNA polymerase potentially can carry out a dNTP degradation reaction (26, 27). All components in Enzyme I- and Enzyme II-catalyzed poly(dA) replication reactions were monitored for relatively long periods of reaction time, and the results are presented in Fig. 6. This experiment shows that in the reaction catalyzed by Enzyme I, all thymidylate residues disappearing from the triphosphate form are recovered in the polymer. In the reaction catalyzed by Enzyme II, the thymidylate residues disappearing from the triphosphate can be accounted for by summing the thymidylate residues in the polymer form and in the monophosphate form. It should be noted that conversion of dTTP to dTMP did not go through a lag phase, suggesting that the 3'-terminal nucleotide of the growing chain was competed for continuously by the polymerase and nuclease functions of the enzyme. The conversion continued when replication of all template nucleotides was complete. The lower net incorporation of the monomer for Enzyme II is also consistent with "continuous competition" since the template chains would also be expected to be susceptible to the action of the 3'-exonuclease. It is interesting to note that in the presence of dTTP the 3'-exonuclease of Enzyme II appeared to be stimulated since the rate of conversion of dTTP to dTMP was twice the hydrolysis rate in the absence of dTTP (Table IV). The properties of yeast DNA polymerase II in dNTP degradation reactions are similar to those reported for T, DNA polymerase (27) but differ from those catalyzed by Escherichia coli polymerase I (26) and human KB cell DNA polymerase-β (13).

Pyrophosphate Exchange and Pyrophosphorolysis—Both yeast DNA polymerases carried out pyrophosphate exchange and pyrophosphorolysis reactions. Bacterial DNA polymerases and mammalian DNA polymerase a are all capable of carrying out these reverse reactions of nucleotide polymerization in the presence of pyrophosphate (1, 2, 12, 13). DNA polymerase β, on the other hand, does not catalyze these reactions to any detectable extent (12, 13).
Yeast DNA Polymerases

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FIG. 5. Proofreading exonuclease in yeast DNA polymerases. Reactions were carried out in the presence of 50 mM Tris/Cl buffer at pH 8.0, 2.5 mM MgCl$_2$, 2 mM 2-mercaptoethanol, 0.2 mM [2-$^3$H]dTTP at 4,200 cpm/nmol, 0.1 mM d(pA)$_7$ (nucleotide concentration), 0.02 mM d(pT)$_2$[methyl-$^3$H]d(pT)$_3$ (thymidylate concentration) or d(pT)$_2$[5-$^3$H]d(pC)$_3$ (thymidylate concentration) at 330 cpm/pmol of [3H]d(pT)$_7$ and 220 cpm/pmol of [3H]d(pC)$_3$, 100 µg/ml of bovine serum albumin, and 70 units of yeast DNA polymerase I or 36 units of yeast DNA polymerase II in a final volume of 200 µl. Progress of each reaction was monitored by removing aliquots (15 µl) at various times and determining $^3$H-nucleotide remaining and 14C-nucleotide polymerized by double label counting procedures.

The pyrophosphate exchange reactions can be easily demonstrated for yeast DNA polymerases when the reactions are carried out in potassium phosphate buffer. The ratio of polymerization to pyrophosphate exchange for yeast Enzyme I (Table IV) was significantly higher than for calf thymus DNA polymerase o and E. coli polymerase I (12). The ratio for yeast Enzyme II (Table IV) was comparable to calf thymus DNA polymerase o and somewhat higher than E. coli polymerase I. The affinity of the yeast enzymes for pyrophosphate measured under exchange reaction conditions was found to be at least 100-fold lower than the individual dNTPs (Table II). The $K_m$ values for pyrophosphate for both enzymes were about equal.

The pyrophosphorolysis reactions were more difficult to demonstrate for the yeast enzymes because the ratios of polymerization activity to pyrophosphorolysis activity are high (Table IV). High concentrations of enzymes and high specific activity pyrophosphate were needed to demonstrate these reactions. The affinity of yeast Enzyme I for pyrophosphate under pyrophosphorolysis reaction conditions was 2.5 times less than the affinity of yeast Enzyme II for pyrophosphate (Table IV).

A summary of the associated enzyme activities for the yeast DNA polymerases is presented in Table IV.

**Table IV**

| Associated enzyme activities of yeast DNA polymerases |
|------------------------------------------------------|
| Enzyme I | Enzyme II |
|-----------------|-----------------|
| Polymerization  | 1.0             | 1.0             |
| dNTP degradation$^a$ | 0.4             |                  |
| 3'-Exonuclease$^a$ | 0.2             |                  |
| Pyrophosphorolysis$^a$ (2 mM) | $3.7 \times 10^{-4}$ | $7.3 \times 10^{-4}$ |
| Pyrophosphate exchange$^a$ (2 mM) | $1.5 \times 10^{-2}$ | $0.14$ |

$^a$ The ratio of polymerization to dNTP degradation was obtained from the same reaction. See legend to Fig. 6 and "Materials and Methods" for details.

DISCUSSION

The principle purpose of this investigation was to compare the properties of two DNA polymerases obtained after extensive purification from commercial yeast cells. The chromatographic profiles of enzyme activity obtained from commercial cells were similar to those of wild type yeast cells. Biochemical characterization showed numerous differences in reaction properties and associated enzyme activities of the two enzymes. Immunological studies showing no neutralization cross-reactivity between the two DNA polymerase activities provide additional evidence for the presence of at least two distinct species of enzyme in yeast.

The results obtained in this study are comparable to those obtained by others and provide some extension of earlier work. The absolute level of total DNA polymerase activity present in the crude extract described here was about 300 units/g of cells.
wet weight. This level is comparable to the 300 to 600 units/g of yeast previously reported by Wintersberger (6, 28). The relative levels of Enzymes I and II appear to be quite different. In the preparations described in this communication, Enzyme II accounted for less than 10% of the total enzyme activity in the crude extract. In the preparation described by Wintersberger (6) Enzyme II accounted for about 30% of the total enzyme activity in the crude extract. The difference in the levels of Enzyme II could be accounted for by differences in the reaction conditions used or by the methods used for extraction of the enzyme.

The total amount of DNA polymerase activity found in commercial yeast cells appears to be somewhat greater than that in wild type yeast cells grown in the laboratory. This apparent difference is due mostly to the difference in water content of cell pellets and commercial yeast. When the levels present in lyophilized preparations were compared, the laboratory grown wild type cells contained 2,600 units/g while commercial yeast had 4,700 units/g. Although the higher level of enzyme activity in commercial cells is somewhat surprising, this finding is consistent with the observation of DNA polymerase levels in Dictyostelium discoideum. In D. discoideum, DNA polymerase level was found to be higher in stationary cells than log phase cells (10). A trivial alternative explanation could be that more efficient extraction of the commercial yeast was obtained.

The enzyme preparations described in this communication are not homogeneous. The specific activities of the preparations obtained in this laboratory were 4- to 10-fold higher for Enzyme I and 2- to 3-fold higher for Enzyme II when compared to Wintersberger's preparations. Although Enzyme II is not homogeneous, the data presented strongly suggest that the DNA polymerase and 3'-exonuclease functions are on the same enzyme protein. A definitive statement concerning the relationship of these two enzyme activities awaits the availability of homogeneous yeast DNA polymerase II.

The biological roles of the two yeast DNA polymerases are unknown at the present. It is interesting to note that in yeast where DNA polymerase β is absent (5–7), yeast DNA polymerase II with error-correcting capability can be found. The role of DNA polymerase β in mammalian cells has been postulated to be in DNA repair and the role of DNA polymerase α has been postulated to be in DNA replication (3). Whether yeast DNA polymerase I indeed has a biological role similar to DNA polymerase α and yeast DNA polymerase II has a role similar to DNA polymerase α remain to be demonstrated. The genetic makeup of yeast is well defined and a number of DNA synthesis and DNA repair mutants are available (29, 30). A detailed biochemical and immunological characterization of yeast DNA polymerases should facilitate the studies on the biological roles of these enzymes and should also aid in our understanding of the molecular defects of these mutants.

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L M Chang

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