DNA Polymerases from Bakers’ Yeast*

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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 iDNA polymerases do not exhibit this property (12, 13). Earlier studies by Wintersberger (6) and Hoffman (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 baker’s yeast (obtained from National Brands Inc., Baltimore, Md.) was used for the purification of the DNA polymerases.

Substrates

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

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18. Oligoribonucleotides were prepared by digestion of polyribonucleotides with micrococcal nuclease and *Escherichia coli* alkaline phosphatase and purified by DEAE-cellulose chromatography as previously described (19). Calf thymus DNA and bovine serum albumin were purchased from Worthington Biochemical Corp. Polyribonucleotides and poly(dA-dT) were purchased from Miles Laboratories. Radioactive dNTPs and [3H]-labeled sodium pyrophosphate were purchased from New England Nuclear. All other reagents were commercial reagent grade.

**Enzyme Assays**

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 dATP, dCTP, and dGTP, and 0.2 mM [3H]-labeled radioactive dTTP 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 initiator nucleotide, 0.05 M Tris/Cl at pH 8.0, 2 mM 2-mercaptoethanol, 0.2 mM complementatory 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. The nucleases were assayed in the nuclease assay were 0.12 mM and 0.18 mM 5'-labeled polymer. Nucleic acid was measured by the decrease of acid-insoluble radioactive activity. One nuclease unit is defined as 1 nmol of polymer nucleotide hydrolyzed per h.

**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 1 M Tris/chloride buffer at pH 8.8, 1 mM potassium phosphate buffer at pH 8.8, 1 mM EDTA, and 10% glycerol. The dialyzed fraction was collected by centrifugation and redissolved in 20 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. 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 DE51 cellulose column (Whatman (0.9 x 28 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 0.05 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 DE51 cellulose column (Whatman (0.9 x 28 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 2: Proteolytic 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 then clarified by centrifugation for 30 min at 8000 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 resuspended by resuspension in about 230 ml of 25 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 0.5 mM EDTA. The precipitate was collected by centrifugation for 30 min at 4°C, washed with the same buffer and 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 Godwin (21). The focusing column consisted of a 10-m1 10 to 30% linear sucrose gradient containing protein to be analyzed, 10% ethylene glycol, and 0.1% amylol with a pH range of 3.5 to 10 (LKDS Produktverkauf A. B., Stockholm). For the nuclease assay it was done in 0.5 M Tris/Cl at pH 8.0. The sucrose gradient was fractionated into 200 fractions and collected in tubes containing 25 µl of solution of 0.75 M potassium phosphate at pH 7.2, 5.5 mg/ml of bovine serum albumin, and 5 mM 2-mercaptoethanol. DNA polymerase and nucleic acid in aliquots of these fractions were dissected as above described. The 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.

**Preparation of Antiserum**

Antiserum to yeast DNA polymerase I was obtained from 2 mg 4-kd 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 (150 µg of 4-kd yeast DNA polymerase I preparation (0.5 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 (equivalent to 2 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 M EDTA, 0.12 M KCl, 1% dimethylsulfoxide, and 0.5 mM phenylmethylsulfonyl fluoride. All operations were carried out at about 4°C. The lysate was clarified by centrifugation and 1/10 volume of 1 M Tris/Cl at pH 8.0 was added to the 24,000 g x g supernatant. The nucleic acids in the supernatant were removed by precipitation with 0.2% protamine sulfate. The precipitation was collected by centrifugation, redissolved, and dialyzed against 0.05 M potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, 0.1 M EDTA, and 10% glycerol. The dialyzed fraction was further purified in a chromatography column (Whatman (0.9 x 28 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 DE51 cellulose column (Whatman (0.9 x 28 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. The volume of the dialysate, Fraction III, was 1350 ml.

**Step 4: Phosphocellulose Column Chromatography—Fraction III**

The column was diluted with 2700 ml of 50 mM potassium phosphate at pH 7.2 and 5 mM 2-mercaptoethanol and loaded onto a phosphocellulose column (4.5 x 45 cm) previously equilibrated with the same buffer. The column was washed with 3 liters of the same buffer, and proteins were eluted with 7.6 liters of a 0.5 M linear gradient of KCl in 50 mM potassium phosphate at pH 7.2 and 5 mM 2-mercaptoethanol. Active fractions (1820 ml) were pooled and labeled Fraction IV. To concentrate this fraction, it was precipitated at 60% (NH₄)₂SO₄ saturation, collected by centrifugation, redissolved in 20 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol, and dialyzed against 2 changes of 2 liters of the same buffer. The final volume of concentrated Fraction IV was less than 100 ml.

**Step 5: DE51 Cellulose Column Chromatography—Fraction IV**

The column was diluted with 2700 ml of 50 mM potassium phosphate at pH 7.2 and 5 mM 2-mercaptoethanol and loaded onto a DE51 cellulose column (2.6 x 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. Two peaks of activity were obtained from this column (cf. Fig. 1). Active fractions eluting...
between 0.06 M and 0.15 M KCl (containing Enzyme I) were pooled and labeled Fraction Va. Active fractions eluting between 0.24 M and 0.32 M KCl (containing Enzyme II) were pooled and labeled Fraction Vb. Both Fractions Va and Vb were precipitated by dialysis to 60% ammonium sulfate saturation in 50 mM potassium phosphate at pH 7.2 and 5 mM 2-mercaptoethanol (16). The protein precipitates were collected by centrifugation and redissolved in 50 mM KCl in 50 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol (8 ml for Fraction Va and 4 ml for Vb).

Step 6: Gel Filtration on Sephadex G-200—Fractions Va and Vb were fractioned on Sephadex G-200 columns (2.6 x 100 cm) equilibrated with 50 mM KCl in 50 mM potassium phosphate (pH 7.2), 5 mM 2-mercaptoethanol, and 10% glycerol. Both enzyme activities were retarded on the Sephadex gel filtration columns and the bulk of the protein appeared at the void volume. The active fractions from the Va column were pooled and labeled Va I and the Vb column fractions were labeled Vb I.

Step 7: Chromatography on Hydroxylapatite—Fraction Va I was loaded directly onto a hydroxylapatite column (1.5 x 10 cm) (23) previously equilibrated with 50 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 200-ml linear gradient of 0.05 M to 0.2 M potassium phosphate at pH 7.2 in 5 mM 2-mercaptoethanol and 10% glycerol. All enzyme activity eluted from the column as a symmetrical peak at 0.15 M potassium phosphate. The active fractions were pooled and labeled Fraction VIIa.

Fraction Vb I was fractionated on a hydroxylapatite column (1 x 4 cm) as described for Fraction Va I except that a 200-ml gradient was used. Two sharp peaks of DNA polymerase activities were eluted from the column. The peak eluting at 0.09 M potassium phosphate is Enzyme II, and the peak eluting at 0.15 M is Enzyme I in Fraction Vb I. Active fractions of Enzyme II were pooled and labeled Fraction VIIb.

Both Fractions VIIa and VIIb were dialyzed overnight against 50 mM KCl in 50 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol.

Step 8: Denatured DNA-Cellulose Column Chromatography—Dialyzed Fraction VIIa was loaded onto a denatured calf thymus DNA-cellulose column (1.5 x 8 cm) (24) equilibrated with 50 mM KCl, 50 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 150-ml linear gradient of 0.05 M to 0.2 M KCl in 0.05 M potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 10% glycerol. DNA polymerase activity eluted from the column as a single peak at 0.15 M KCl. Active fractions were pooled and labeled Fraction VIIIa.

The dialyzed Fraction VIIb was fractionated on a denatured DNA-cellulose column (0.9 x 5 cm) as described for Fraction VIIa except that a 50-Ml gradient was used. DNA polymerase activity was eluted from the column in a single peak at 0.11 M KCl. Active fractions were pooled and labeled Fraction VIIIb.

Fractions VIIIa and VIIIb were dialyzed against 50 mM potassium phosphate at pH 7.2, 5 mM 2-mercaptoethanol, and 50% glycerol. The enzyme preparations were stored at -20°C and the activities were stable for over 2 months.

RESULTS

DNA Polymerases in Wild Type Yeast Cells

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 KCl, 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 KCl, 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 8,000- 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 A280/A260 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-
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 in 50 mM Tris/Cl buffer were 91 and 79% of their corresponding activities at optimum. The magnesium catalyzed rates. When a synthetic template system, such as d(pA)₂₅. d(pT)₅₀, 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. Manganese ion at 0.15 to 0.5 mM catalyzed this reaction at about 10% of the optimum magnesium ion concentration was 6 to 20 mM. Manganese ion at 0.6 to 1.2 mM catalyzed this reaction at about 10% of the optimum magnesium catalyzed rates. When activated DNA was 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 Ammediol/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. Manganese ion at 0.6 to 1.2 mM catalyzed this reaction at about 10% of the magnesium catalyzed rates. When a synthetic template system, such as d(pA)₂₅. d(pT)₅₀, was used as template, magnesium remained the preferred divalent cation. Enzyme I and Enzyme II have sharp magnesium optima at 3 μM and 2 μM, respectively. Manganese ion at 0.15 to 0.5 mM catalyzed the homopolymer replication reactions at about 10% of the optimum magnesium catalyzed rates.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (units/mg)</th>
<th>Specific activity</th>
<th>Total enzyme activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>590,000</td>
<td>9.8</td>
<td>5.78 x 10^6</td>
</tr>
<tr>
<td>II. Protamine sulfate supernatant</td>
<td>288,000</td>
<td>20.0</td>
<td>4.16 x 10^6</td>
</tr>
<tr>
<td>III. Dialyzed (NH₄)₂SO₄</td>
<td>72,400</td>
<td>47.8</td>
<td>3.46 x 10^6</td>
</tr>
<tr>
<td>IV. Phosphocellulose</td>
<td>2,500</td>
<td>648</td>
<td>1.62 x 10^6</td>
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</tbody>
</table>

**Properties of Yeast DNA Polymerases**

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 Ammediol/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. Manganese ion at 0.6 to 1.2 mM catalyzed this reaction at about 10% of the magnesium catalyzed rates. When a synthetic template system, such as d(pA)₂₅. d(pT)₅₀, was used as template, magnesium remained the preferred divalent cation. Enzyme I and Enzyme II have sharp magnesium optima at 3 μM and 2 μM, respectively. Manganese ion at 0.15 to 0.5 mM catalyzed the homopolymer replication reactions at about 10% of the optimum magnesium catalyzed rates.

**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.

![Fig. 2. Effects of antiserum to yeast DNA polymerase I on yeast DNA Polymerases.](http://www.jbc.org/) One unit each of yeast DNA polymerase I (Fraction VIIIa) and DNA polymerase II (Fraction VIIIb) was incubated with serial dilutions of the control serum and antiserum (diluted with 0.015 M potassium phosphate buffer at pH 7.2 containing 0.15 M NaCl) to yeast DNA polymerase I for 16 h at 4°C in a total volume of 20 μl. DNA polymerase activity remaining after the antiserum treatment 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.

![Graph of Enzyme Activity vs. Dilution of Antiserum](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Yeast DNA Polymerases</th>
<th>Specific Activity</th>
<th>Total Enzyme Activity (units)</th>
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<tbody>
<tr>
<td>Fraction VIIIa</td>
<td>340,000</td>
<td>0.23 x 10⁶</td>
</tr>
<tr>
<td>Fraction VIIIb</td>
<td>119,000</td>
<td>5.48 x 10⁵</td>
</tr>
</tbody>
</table>

**Results:**

- The purification of yeast DNA polymerases was successful. The best preparation of Enzyme I (specific activity was 650,000 units/mg) was obtained from the DE11 column as a split peak. No heterogeneity was observed when Enzyme I was fractionated on hydroxylapatite or denatured DNA-cellulose. The Enzyme II fraction eluted from the DE11 column still contained 40 to 70% of Enzyme I which can be completely removed from Enzyme II on the hydroxylapatite column (Fig. 3A).

A rigorous study of the purity of the enzyme preparations obtained have 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(pApApApT). 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 polymer(dA) replication 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 oligohymidylic acid initiated poly(rA) in the presence of magnesium ion. When magnesium ion was used, however, the rates of replication of poly(dA) and poly(rA) were found to be almost identical for Enzyme I. Oligoriboadenylate was found to be an effective initiator for poly(dT) replication in the presence of either magnesium ion or manganese ion for Enzyme I. Enzyme II can also utilize oligoriboadenylate-initiated poly(dT) template, although at a much lower rate. Oligouridylate-initiated poly(dA) was found to be a poor template system for Enzyme I and is ineffective with Enzyme II.

**Table II**

<table>
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<th>Substrate affinities of yeast DNA polymerases</th>
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<td>Reaction</td>
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<td>Enzyme I</td>
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| Polymerization | Activated calf thymus DNA | 6.0 μg/ml | 3.0 μg/ml |
| dATP | 3.7 μM | 3.7 μM |
| dGTP | 2.0 μM | 1.9 μM |
| dCTP | 6.9 μM | 5.3 μM |
| dTTP | 8.3 μM | 8.1 μM |
| Pyrophosphorylase | Sodium pyrophosphate | 3.8 mM | 1.6 mM |
| Pyrophosphate | Sodium pyrophosphate | 1.2 mM | 1.1 mM |

* The magnesium chloride concentration in DNA primed reactions was 8 mM and in synthetic template primed reactions was 2.5 mM.

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 have identical heat inactivation kinetics.

**Table III**

<table>
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<th>Template specificities of yeast DNA polymerases</th>
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<td>Template</td>
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<td>Mg*++</td>
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<tbody>
<tr>
<td>Enzyme I</td>
<td>17.0</td>
<td>2.0</td>
<td>4.4</td>
<td>0.41</td>
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<td></td>
<td></td>
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<tr>
<td>Enzyme II</td>
<td>16.0</td>
<td>2.0</td>
<td>4.4</td>
<td>0.41</td>
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* The magnesium chloride concentration in DNA primed reactions was 8 mM and in synthetic template primed reactions was 2.5 mM.

The manganese chloride concentration was used as 0.5 mM.

**Deoxynucleotidase Triphosphate Degradation** — The presence
1878 Yeast DNA Polymerases

B

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FIG. 3. Relationship of DNA polymerase and 3'-exonuclease functions in yeast DNA polymerases. Panel A, 4.3 A₂₅₀ 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 A₂₅₀ 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 KC1 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)₂₅₀[d(pA)]₈₅ as substrate for 3'-exonuclease assays. The recovery of enzyme activity for each of the methods of analysis was more than 80%.

tered 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 T₇ 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 α 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

![Graph showing proofreading exonuclease in yeast DNA polymerases](image)

**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 MgCl₂, 2 mM 2-mercaptoethanol, 0.2 mM [2-³²P]dTMP at 4,200 cpm/nmol, 0.1 mM d[³²P]dATP (nucleotide concentration), 0.02 mM d[³²P]dCTP (thymidylate concentration) or 0.02 mM d[³²P]dGTP (thymidylate concentration) at 330 cpm/pmol of [³²P]dATP and 220 cpm/pmol of [³²P]dCTP, 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 ³²P-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 I and E. coli polymerase I (12). The ratio for yeast Enzyme II (Table IV) was comparable to calf thymus DNA polymerase I 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 K₅₀ 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 to pyrophosphorolysis exchange for yeast Enzyme I (Table IV) was significantly higher than for calf thymus DNA polymerase α and E. coli polymerase I (12). The ratio for yeast Enzyme II (Table IV) was comparable to calf thymus DNA polymerase α and somewhat lower 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 K₅₀ 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.

![Graph showing degradation of deoxynucleoside triphosphate by yeast DNA polymerases](image)

**Fig. 6.** Degradation of deoxynucleoside triphosphate by yeast DNA polymerases. The dNTP degradation reactions were carried out as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>TABLE IV</th>
<th>Associated enzyme activities of yeast DNA polymerases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme I</td>
</tr>
<tr>
<td>Polymerization</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP degradation</td>
<td>0.4</td>
</tr>
<tr>
<td>³'-Exonuclease</td>
<td>0.2</td>
</tr>
<tr>
<td>Pyrophosphorolysis</td>
<td>3.7 x 10⁻³</td>
</tr>
<tr>
<td>Pyrophosphate exchange</td>
<td>1.5 x 10⁻⁴</td>
</tr>
</tbody>
</table>

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

* Exonuclease activity was measured using d[pA]₃₂P·[2-³²P]dCTP (8,000 cpm/nmol of thymidylate, the concentrations of deoxyadenylate was 0.2 mM and the concentration of thymidylate was 0.1 mM) as substrate. One unit equals 1 nmoi of [²³⁴C]dCTP, hydrolyzed per h. The polymerization rate used in this calculation equals nanomoles of dTMP polymerized per h using d[pA]₃₂P·[2-³²P]dCTP as template and [methyl-³²H]dATP as monomer. Other conditions for the reactions were identical.

* Polymerization rate used in this calculation represents nanomoles of total nucleotide polymerized per h with activated calf thymus DNA under reaction conditions identical to the pyrophosphorolysis reaction except in the absence of sodium pyrophosphate.

* Polymerization rate used in this calculation represents nanomoles of total nucleotide polymerized per h with activated DNA under reaction conditions identical to the exchange reaction; that is, the presence of 2 mM sodium pyrophosphate.

**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 make-up 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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