Purification and Characterization of DNA Polymerase II from the Yeast Saccharomyces cerevisiae

IDENTIFICATION OF THE CATALYTIC CORE AND A POSSIBLE HOLOENZYME FORM OF THE ENZYME

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We have purified yeast DNA polymerase II to near homogeneity as a 145-kDa polypeptide. During the course of this purification we have detected and purified a novel form of DNA polymerase II* that we designate as DNA polymerase II*. The most highly purified preparations of DNA polymerase II* are composed of polypeptides with molecular masses of 200, 80, 34, 30, and 29 kDa. Immunological analysis and peptide mapping of DNA polymerase II and the 200-kDa subunit of DNA polymerase II* indicate that the 145-kDa DNA polymerase II polypeptide is derived from the 200-kDa polypeptide of DNA polymerase II*. Activity gel analysis shows that the 145- and the 200-kDa polypeptides have catalytic function. The polypeptides present in the DNA polymerase II* preparation co-purify with the polymerase activity with a constant relative stoichiometry during chromatography over five columns and co-sediment with the activity during glycerol gradient centrifugation, suggesting that this complex may be a holoenzyme form of DNA polymerase II*. Both forms of DNA polymerase II possess a 3'-5' exonuclease activity that remains tightly associated with the polymerase activity during purification. DNA polymerase II* is similar to the proliferating cell nuclear antigen (PCNA)-independent form of mammalian DNA polymerase δ in its resistance to butylphenyl-dGTP, template specificity, stimulation of polymerase and exonuclease activity by KCl, and high processivity. Although calf thymus PCNA does not stimulate the activity of DNA polymerase II* on poly(dA):oligo(dT), possibly due to the limited length of the template, the high processivity of yeast DNA polymerase II* on this template can be further increased by the addition of PCNA, suggesting that conditions may exist for interactions between PCNA and yeast DNA polymerase II*.

DNA polymerases have a central role in the replication and repair of cellular DNA. The complexity and importance of these processes to the cell are reflected in the multiple species of DNA polymerases found in both prokaryotic and eukaryotic cells, where specific functions are performed predominantly by one or another species of polymerase (see Ref. 1 for review). Four classes of DNA polymerases (DNA polymerases I, II, III, and mitochondrial) have been described from the yeast Saccharomyces cerevisiae (2-5). Yeast DNA polymerase I (Ypo1 I) is biochemically and structurally similar to DNA polymerase α of higher eukaryotes; it is inhibited by aphidicolin (6) and butylphenyl-dGTP (BuPdGTP) (7); it can be isolated as a complex with an associated primase activity (8); and it lacks exonuclease activity (2). DNA polymerase II (Ypo1 II) is immunologically (2) and genetically (9) distinct from Ypo1 I. Characterizations of partially purified Ypo1 II have shown that it differs significantly from Ypo1 I in possessing an associated 3'-5' exonuclease activity (2). DNA polymerase III (Ypo1 III) is similar to Ypo1 II in having an associated 3'-5' exonuclease activity (5), but it is immunologically and genetically (9) distinct from Ypo1 I. Ypo1 II and Ypo1 III have properties similar to those of mammalian δ DNA polymerases because of their associated exonuclease activity and resistance to BuPdGTP (7). The mitochondrial DNA polymerase, while not extensively studied, differs from the nuclear polymerases in its high Mg2+ requirement and its smaller size (4).

Results with the SV40 in vitro replication system (10, 11) as well as studies with permeabilized mammalian cells (12, 13) have indicated that more than one DNA polymerase species is involved in replicative DNA synthesis. This has recently been confirmed in the yeast system where the genes for Ypo1 I and Ypo1 III have been identified (14-17) and have been shown to be required for DNA replication (18-20). The ability in yeast to augment biochemical studies with classical genetic and molecular genetic approaches makes the yeast system an attractive one for analyzing the in vivo functions of enzymatic activities for serving as a model system for eukaryotic cells.

In this paper we describe the purification and characterization of DNA polymerase II*, whose role in the cell remains to be determined. For comparative purposes, we have also included in this paper the purification of Ypo1 I-primase complex and Ypo1 III. Ypo1 II has been partially purified by others with estimates of 100-150 kDa for its size (2, 3, 9, 21). Our purest preparations of Ypo1 II contain a polypeptide of 145 kDa as the major species, in close agreement with the earlier

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estimates. During the purification of yPol II, another peak of polymerase activity could be resolved from yPol I, II, and III. This polymerase species was purified and consists of five polypeptides of 200, 80, 34, 30, and 29 kDa that co-elute with the activity and may represent an assembly of subunits into a complex. We present physical evidence showing that the 145-kDa yPol II polypeptide is derived from the 200-kDa polypeptide of this DNA polymerase. The complex containing the 200-kDa polypeptide may therefore be a holoenzyme form of yPol II, and we have designated it yPol II*. We present biochemical characterizations of yPol II and yPol II* that address some of the similarities and the differences between the various yeast polymerases as well as the similarities to higher eukaryotic polymerases.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Purification of DNA Polymerases**—Five peaks of DNA polymerase activity could be resolved by chromatography on a Mono Q anion exchange column of fractions enriched for DNA polymerases (Fig. 1). As described under "Experimental Procedures," yPol III was purified from pool A; yPol I-primase complex was purified from pool C; yPol II was purified from pool D; and the polymerase species yPol II* was purified from pool E. Western blot analysis of pool B indicated that it was a mixture of yPol I and degraded yPol III (data not shown), and no further characterization was done of pool B DNA polymerases. Previous purifications of yeast DNA polymerases have used DEAE-anion exchange column chromatography to resolve DNA polymerase I, II, and III (2, 3, 5, 9), so our results with the Mono Q column are not directly comparable but are similar to the previous purifications in having yPol II eluting after yPol I and yPol III. A major difference, however, is that we have consistently detected two peaks of polymerase activity (peaks D and E in Fig. 1) eluting after the yPol I-primase complex.

The purification of yPol II and yPol II* is summarized in Table I. The polymerase activity using poly(dA):oligo(dT) as the substrate could not be measured accurately in fractions I and II, therefore, the amount of activity present in fraction IV, when the different polymerases were resolved, was taken as 100%. The yPol II and yPol II* fractions contained an exonuclease activity that co-eluted exactly with the polymerase activity on the Mono Q column as well as the four subsequent columns (data not shown). The ratio of polymerase to exonuclease activity remains approximately constant throughout the purification for yPol II and yPol II*, although it differs for the two polymerases. For yPol II, a 100-fold purification of the polymerase activity and a 74-fold purification of the nuclease activity was achieved. For yPol II*, a 22-fold purification of polymerase activity and a 17-fold purification of nuclease was achieved from the Mono Q pools.

The polypeptide composition of the most purified polymerase fractions was determined by SDS-polyacrylamide gel electrophoresis and is shown in Fig. 2. The yPol I-primase complex, as purified by the procedure described here, has the expected 70-kDa subunit and the 58- and 48-kDa primase subunits but, instead of a ladder of polypeptides between 140-180 kDa (8), has an intact 180-kDa catalytic subunit. Although we have tried to minimize proteolysis by the liberal use of protease inhibitors and the use of a PEP4 disruption.

2 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopics are included in the microfilm edition of the Journal that is available from Waverly Press.

![Fig. 1. Mono Q chromatography of yeast DNA polymerases.](http://www.jbc.org/)

Yeast protein fraction enriched for DNA polymerase activity was isolated and chromatographed on a Mono Q HR 16/10 column as described under "Experimental Procedures." Aliquots of column fractions were assayed for DNA polymerase activity using activated calf thymus DNA (O-O) or poly(dA):oligo(dT) (o-o) as templates. The indicated fractions were pooled, yPol III was purified from pool A; yPol I-primase complex was purified from pool C; yPol II was purified from pool D; and yPol II* was purified from pool E.
Immunological Relatedness of Yeast DNA Polymerases—Antibodies raised against the yeast DNA polymerases were used in Western blots to probe the immunological relatedness of the purified DNA polymerases. As shown in Fig. 4, antibody to the 180-kDa catalytic subunit of yPo1 I is specific to this subunit of yPo1 I and does not recognize any of the other three DNA polymerases. The antibody raised against yPo1 III recognizes only the polypeptides present in the yPo1 III fraction and does not react with any of the other three DNA polymerases. Identical results were obtained using the rabbit antiserum against yPo1 III obtained from Dr. Peter Burgers (Washington University, St. Louis, MO) (data not shown). The antibody against yPo1 II* reacts to all of the polypeptides present in the yPo1 II* fraction except for the smallest 29-kDa subunit. The antibody to yPo1 II does not recognize either yPo1 I or yPo1 III but as a significant reaction to the yPo1 II polypeptide. Although an equivalent mass of protein was loaded within each sample well, on a molar basis we estimate that there is twice as much of the 145-kDa polypeptide as there is the 200-kDa polypeptide of yPo1 II*. Despite this molar excess, the staining intensity of the yPo1 II polypeptide is always less than for the 200-kDa polypeptide.

Partial Proteolysis of yPo1 II and yPo1 II*—One possible explanation for the reaction of the 145-kDa yPo1 II polypeptide to the yPo1 II* antiserum is that yPo1 II is derived by proteolysis from the 200-kDa catalytic subunit of yPo1 II*. The proteolysis could remove antigenic determinants resulting in the loss of immunoreactivity of yPo1 II observed with the antiserum. To test this hypothesis, we analyzed the peptide products resulting from partial proteolysis of yPo1 II and the isolated 200-kDa subunit of yPo1 II*. The products from digestion with Staphylococcus aureus V8 protease and Arg-C protease were analyzed by SDS-polyacrylamide gel electrophoresis and were visualized by silver staining (Fig. 6A) or Western blotting and probing with yPo1 II* antiserum (Fig. 5B). V8 protease digestion, which cleaves after glutamic and aspartic acid residues, generated many proteolytic fragments, and approximately half of these from yPo1 II and the 200-kDa polypeptide co-migrate (Fig. 5A, lanes 3 and 4). Digestion with Arg-C protease did not produce as many proteolytic fragments, but several co-migrating bands can be observed (Fig. 5A, lanes 5 and 6). The polypeptides that co-migrate are indicated by a | between lanes 3 and 4 and between lanes 5 and 6. Western blot analysis of these fragments indicates that only the larger proteolytic fragments of the 200-kDa polypeptide are still recognized by the antibody to yPo1 II* (Fig. 5B, lanes 4 and 6) and that many of these fragments co-migrate with antigenic fragments of yPo1 II (Fig. 5B, lanes 3 and 5). A possible N- or C-terminal fragment of the 200-kDa polypeptide that is strongly antigenic but is absent from the digestion of yPo1 II is indicated by an asterisk in Fig. 5B. We conclude from these data that yPo1 II is a lower molecular mass form of the 200-kDa polypeptide of yPo1 II*.

Glycerol Gradient Centrifugation and Gel Filtration of Yeast DNA Polymerases—In order to obtain data on the physical sizes of the yeast DNA polymerase species, the highly purified fractions were subjected to glycerol gradient centrifugation. The polymerase activities of yPo1 I, II, and II* and the nuclease activities of yPo1 II and II* were measured in the gradient fractions and are shown in Fig. 6. By comparison to the sedimentation of the standard proteins, the sedimentation coefficients of yPo1 I, yPo1 II, and yPo1 II* are 7.8, 5.8, and 9.0 S, respectively.

The association between the polymerase and exonuclease activities of yPo1 II and yPo1 II* is very tight. These activities co-purify exactly over five column fractions (data not shown) and co-sediment in glycerol gradients (Fig. 6, B and C). For yPo1 II, the 145-kDa polypeptide that is the major component of yPo1 II also co-sediments with the polymerase and exonuclease activity (data not shown) suggesting that both activities reside in the same polypeptide.

It is generally accepted that yPo1 I and the higher eukaryotic α polymers exist as a complex of polypeptides with an associated primase activity (31). SDS-polyacrylamide gel electrophoresis of glycerol gradient fractions containing yPo1 I show that the four polypeptides of the yPo1 I-primase complex (Ref. 8 and Fig. 2) all co-sediment with the polymerase activity (Fig. 6D). The five polypeptides comprising yPo1 II* (Fig. 2) also behave as subunits of a complex as shown by their co-sedimentation with the polymerase activity (Fig. 6E).

Analytical gel filtration using the Pharmacia Superose 6 HR 10/30 column was performed on yPo1 I, II, and II* in order to determine their Stokes radii. The Stokes radius was determined by comparison to standard proteins and was used in conjunction with the sedimentation coefficient obtained from glycerol gradient centrifugation to calculate the molecular masses and frictional coefficients of yPo1 I, yPo1 II, and yPo1 II* by the method of Siegel and Monty (32). The results are summarized in Table II and indicate that yPo1 I, yPo1 II, and yPo1 II* are highly asymmetric and, at the ionic strength used in this analysis, behave as monomers or monomer complexes (i.e. contain only one catalytic subunit in the complex).

Reaction Requirements—Both yPo1 II and yPo1 II* polymerase activities on activated calf thymus DNA were stimulated by KCl. As shown in Fig. 7, maximal stimulation occurred at
Fig. 2. SDS-polyacrylamide gel electrophoresis of purified yeast DNA polymerases. 1.5 μg of yPo1 I (fraction V-C), 1.1 μg of yPo1 III (fraction VII-A), 0.7 μg of yPo1 II (fraction VIII-D), and 1.5 μg of yPo1 II* (fraction VIII-E) were electrophoresed in a 10% polyacrylamide gel with a 5% stacking gel and visualized by silver staining. The migration of the molecular weight markers (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase) are indicated by the arrows on the right.

100 mM KCl for yPo1 II and at 125 mM KCl for yPo1 II*. This stimulation by KCl is similar to results obtained with the HeLa cell DNA polymerase δ (33). When poly(dA):oligo(dT) was used as template primer, maximal stimulation was at 50 mM KCl for both DNA polymerases while KCl concentrations greater than 100 mM were inhibitory (not shown). The exonuclease activity of both yPo1 II and yPo1 II* were also stimulated by KCl as shown in Fig. 7, A and B.

Both DNA polymerases required Mg++ for activity with yPo1 II having an optimum Mg++ concentration of 6 mM and yPo1 II* having a broad Mg++ optimum of 6–10 mM (Fig. 7, C and D). Although the stimulation of DNA polymerase activity by KCl is similar to the behavior of the mitochondrial DNA polymerase from Drosophila melanogaster (26), yPo1 II and yPo1 II* have Mg++ optima substantially less than the 50 mM Mg++ of the yeast mitochondrial DNA polymerase (4).

The Kₘ values for dNTPs and DNA substrates were determined for yPo1 I, yPo1 II, and yPo1 II* at their optimal KCl concentration and are shown in Table III. The Kₘ values for dNTPs are comparable with each other and with previously measured values for yeast DNA polymerases (2, 3, 7). The differences in Kₘ values for yPo1 II and yPo1 II* are not significant. The Kₘ for activated calf thymus DNA of yPo1 I is somewhat higher than published values and may result from variations between different stocks of this substrate. The exonuclease activities of yPo1 II and yPo1 II* have Kₘ values for single-stranded DNA substrate that is somewhat higher than, but comparable to, the 1.5 μg/ml value for yPo1 III (7).

Inhibitor Studies—The inhibition of DNA polymerase activity by various inhibitors can be diagnostic for a particular DNA polymerase species. Aphidicolin, for example, inhibits α and δ DNA polymerases while it has no effect on β or mitochondrial DNA polymerases (1). BuPdGTP has been used to distinguish β from δ DNA polymerase based on the relative resistance of δ DNA polymerase species to this analogue (34). Because yPo1 II and yPo1 II* are similar to the δ DNA polymerases in having an associated nuclease activity, it was of interest to determine if they were also resistant to BuPdGTP. Shown in Fig. 8 are inhibition curves of the four yeast DNA polymerase species. All four DNA polymerases
FIG. 4. Western blots against purified yeast DNA polymerases. 70 ng of the most pure fractions of yPol I, yPol II, yPol III, and yPol II* were subjected to electrophoresis in a 4–20% polyacrylamide gel, transferred to Immobilon PVDF membrane, and immunoblotted with the indicated yPol antisera. The migration of prestained molecular weight markers (Bethesda Research Laboratories) is indicated on the left (myosin, phosphorylase b, bovine serum albumin, ovalbumin).

FIG. 5. Partial proteolytic digestion of yPol II and the 200-kDa subunit of yPol II*. Proteolytic digestions of 2 μg of yPol II (lanes 3 and 5) or 2 μg of the 200-kDa subunit of yPol II* (lanes 4 and 6) with either S. aureus V8 protease (lanes 3 and 4) or Arg-C protease (lanes 5 and 6) were performed as described under "Experimental Procedures." Lanes 1 and 2 contain undigested yPol II and the 200-kDa polypeptide, respectively. The samples were electrophoresed on a 10–20% polyacrylamide gel and either silver-stained (A) or analyzed in Western blots with the antiserum to yPol II* (B). Co-migrating polypeptides are indicated by a □ between lanes 3 and 4 and 5 and 6 in A and a highly antigenic polypeptide that is present in the 200-kDa polypeptide but missing in yPol II is indicated by an * in B.

Table III summarizes results with the above inhibitors as well as with araGTP and ddTTP. Although BuPdGTP, and to a lesser extent araGTP, can distinguish yPol I from yPol II and yPol II*, none of the inhibitors tested differentiates between yPol II, yPol II*, and yPol III.

Template Utilization by Yeast DNA Polymerases—The ability of the different DNA polymerase species to utilize various DNA substrates may in some cases be diagnostic for a particular species. DNA polymerase γ, for example, is the only cellular polymerase that can use RNA as a template (1). The PCNA-independent pol δ is quite active on a poly(dA) template primed with oligo(dT) (33, 34), whereas the PCNA-dependent pol δ cannot utilize this substrate unless supplemented with PCNA (35). We therefore measured the activity of the purified yeast DNA polymerases on a variety of sub-
Yeast DNA Polymerase II

Fig. 6. Glycerol gradient centrifugation of yeast DNA polymerases. A, 170 units of yPol I-primase complex (fraction V-C); B, 62 units of yPol II (fraction VIII); and C, 63 units of yPol II* (fraction VIII) were centrifuged in glycerol gradients (15–40% in 50 mM Tris-HCl, pH 8, 1 mM EDTA, 200 mM KCl, and 1 mM dithiothreitol) for 13 h at 50,000 rpm in a Beckman SW55 rotor at 4°C. Six drop fractions (145 μl) were collected from the bottom and assayed for DNA polymerase (O–O) or nuclease activity (■–■). Arrows indicate the position and S values of marker proteins (thyroglobulin, catalase, and aldolase). Aliquots of the indicated gradient fractions were electrophoresed in 4–20% polyacrylamide gels and visualized by silver staining: n, gradient fractions from sedimentation of yPol I-primase complex; E, gradient fractions from sedimentation of yPol II*. Migration of marker proteins are indicated on the right (myosin, β-galactosidase, bovine serum albumin, and ovalbumin).

strates. As shown in Table IV, yPol I is most active on activated calf thymus DNA, has no reverse transcriptase activity, prefers ribonucleoside to deoxyribonucleoside primers on a poly(dT) template, and can support synthesis on an unprimed poly(dT) template because of the primase subunits (results consistent with previously published characterizations of yPol I (6) and yPol I-primase complex (8)). The results with yPol III are also consistent with previously published work (5), yPol III can utilize ribo-primers and has no primase activity. The template/primer preferences for yPol II and yPol II* are very similar to each other (Table IV). Like yPol III, they have no reverse transcriptase or primase activity and can utilize ribo-primers. Unlike yPol III, they efficiently utilize poly(dA):(dT)₉₀. The low activity of yPol II on activated calf thymus DNA when the ionic strength is low (Fig. 1) accounts for the earlier reports of the preferential utilization of poly(dA):(dT)₀ over activated calf thymus DNA (2, 3). At close to physiological ionic strengths, however, yPol II and yPol II* utilize activated calf thymus DNA very efficiently and, in the case of yPol II, this becomes the preferred substrate.

Processivity Measurements—The processivity of DNA polymerases is a measurement of the number of nucleotides polymerized by the enzyme after binding to and prior to dissociating from the primer-template. We have measured the processivity of yPol I, yPol II, and yPol II* on poly(dA):(dT)₀. Under reaction conditions where less than 1 pmol of labeled dTMP is incorporated per pmol of primer, the products should represent only one round of binding, extension, and dissociation rather than multiple extensions from the same primer. The processivity may therefore be measured by direct sizing of the products. PCNA affects the processivity of some δ polymerases and thus differentiates them into PCNA-dependent and PCNA-independent classes (33, 36, 37). Calf thymus PCNA has also been shown to stimulate the activity of yPol III by increasing the low processivity of yPol III (38). Because
of the effect that PCNA can have on processivity, we have included PCNA in this analysis. The results are shown in Fig. 9. yPo1 I polymerizes 5–10 nucleotides per synthesis cycle (lane 1). The low processivity of yPo1 I is not affected by calf thymus PCNA (lane 2), in agreement with the results of Burgers (38). In the absence of PCNA, both yPo1 II and yPo1 II* are capable of polymerizing at least 100 nucleotides per synthesis cycle (lanes 3 and 5). In the presence of calf thymus PCNA, the high processivity of both enzymes is further increased (lanes 4 and 6). Unlike the case with yPo1 III (38), little or no stimulation of polymerase activity is observed under these conditions (data not shown), possibly due to the inherent efficient utilization of the poly(dA):oligo(dT) primer-template by yPo1 II and yPo1 II* and to the restricted length of the template (the average size of the poly(dA) is 400 nucleotides). However, while yPo1 II and yPo1 II* are not dependent on PCNA for activity on poly(dA):oligo(dT), they are clearly affected by PCNA. Because PCNA does not bind to DNA (35), the effect of PCNA on the processivity of yPo1 II and yPo1 II* presumably occurs through its interaction with these polymerases.

Characterization of Exonucleolytic Activity—A potent exonuclease activity is tightly associated with yPo1 II and yPo1 II*. Concomitant analysis of the polymerase activity on activated calf thymus DNA and exonuclease activity on [3H]poly(dT) for these two polymerases as well as for T4 DNA polymerase under identical conditions was performed to compare their activities. The polymerase to exonuclease ratio obtained was 9:1 for yPo1 II, 6:1 for yPo1 II*, and 140 for T4 DNA polymerase (data not shown). The potency of the yPo1 II- and yPo1 II*-associated exonuclease is also indicated by the inability to inhibit the activity with up to 5 mM AMP (data not shown), a concentration of AMP which will substantially inhibit the exo-nuclease activities of β DNA polymerases (34, 39) and Escherichia coli PolII (40). To characterize further the exonuclease activity, the directionality and specificity for mismatched termini of the yPo1 II- and yPo1 II*-associated exo-nuclease activities were determined by analysis of the reaction products from excision of mismatched and matched 3’-terminal ends (Fig. 10). The results indicate that the exonuclease-lytic activity occurs in the 3’→5’ direction, since there was no loss of total radioactivity upon counting the bands present in the lanes. No preference for excision of a mismatched base over a matched base pair could be observed by measuring the rates of excision of mismatched or matched bases.

DISCUSSION

Five peaks of DNA polymerase activity from yeast can be resolved by Mono Q chromatography of protein fractions enriched for polymerases (Fig. 1). As described under “Experimental Procedures,” four highly purified DNA polymerase species can be isolated from the Mono Q pools. Previously, anion exchange chromatography had resolved two (2, 3) or three (5, 9) peaks of DNA polymerase activity. The detection of the four DNA polymerase activities described in this paper may be due to the higher resolution achieved by the Mono Q anion exchange column and/or to the successful suppression of proteolysis by using a pep4::URA3 disruption mutant and a mixture of protease inhibitors during the purification. Another factor in detecting and purifying the four DNA polymerases is the use of poly(dA):oligo(dT) as template:primer during purification since yPo1 II and yPo1 II* utilize activated calf thymus DNA inefficiently at low ionic strength. The assignment of the polymerase species isolated from the Mono Q column-pool D (Fig. 1) as yPo1 II is based on the following reasons: (i) the immunological distinction from yPo1 I and yPo1 III (Fig. 4), (ii) its associated exonuclease activity, (iii) close agreement in size of the 140-kDa polypeptide with the estimated size of 150 kDa of partially purified yPo1 II (2, 9). The exonuclease and polymerase activities in the yPo1 II fractions co-purified over five column chromatographies and

Table II

<table>
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<th>DNA polymerase</th>
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<td>76</td>
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* Determined as in Ref. 32.
* ND, not determined.

Fig. 7. Effect of KCl and Mg2+ on yPo1 II and yPo1 II*. Standard polymerase assays using activated calf thymus DNA (C—O) or exonuclease assays (●—●) were performed as described under “Experimental Procedures” except that the concentrations of KCl and Mg2+ were varied as indicated. For the Mg2+ analysis in B and D, the [KCl] was maintained at 100 mM. A and B 0.3 units of yPo1 II. C and D 0.6 units of yPo1 II*.

Table III

<table>
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<th>Comparison of yeast DNA polymerases I, II, and II*</th>
<th>yPo1 I</th>
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<th>yPo1 II*</th>
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<td>Activated calf thymus DNA</td>
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<td>26</td>
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<td>Poly(dT) (exonuclease Km, μg/ml)</td>
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<td>2.6</td>
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<td>0.3</td>
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<td>30</td>
<td>40</td>
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<td>25</td>
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<td>&gt;100</td>
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* The concentration of all four dNTPs was varied together.
× dCTP was decreased to 10 μM.
× dGTP was decreased to 10 μM.
× dTTP was decreased to 10 μM in the regular assay.
co-sedimented in glycerol gradients. The tight association of these two activities suggests that the 145-kDa protein in the yPo1 II preparation (Fig. 2), which we have shown to be catalytically active for polymerase activity (Fig. 3), is also responsible for the exonuclease activity.

Recently, Budd et al. (9) have described the purification of yPo1 II as a 132-kDa polypeptide. The 132-kDa yPo1 II polypeptide is estimated to be an active proteolytic fragment of a 170-kDa polypeptide based on activity gel analysis of yPo1 II. Our yPo1 II is also a metastable proteolytic product of a larger polypeptide, although in our case we see a 145-kDa polypeptide derived from a 200-kDa polypeptide. It may be possible that the 170-kDa polypeptide activity seen by Budd et al. (9) is a proteolytic fragment of the 200-kDa catalytic subunit of yPo1 II*.* The larger sizes that we see for yPo1 II may be due to the use of the protease-deficient PEP4 disruption strain and/or to the faster purification procedure made possible by use of the fast protein liquid chromatography system. Alternatively, the 132- and 145-kDa polypeptides may be equivalent in size, but the mobility may be dependent on conditions of electrophoresis. Budd et al. (9) also observed another peak of polymerase activity that they called peak 2 in their polymerase profile; however, they did not identify this peak. Our peak 2 polymerase sediments as a 132-kDa globular protein whereas yPo1 II* behaves as a complex of subunits of 200, 80, 34, 30, and 20 kDa. and activity gel analysis shows catalytically active polypeptides of different molecular masses.

One major difference between our yPo1 II and the previously purified yPo1 II is that stimulatory factors (9) are not required by us to detect yPo1 II activity, even for glycerol gradient or gel filtration fractions. Although most of the columns used are not identical for the two purifications of yPo1 II, some columns are similar and would have separated at least stimulatory factor 1 (flow through of cation exchange column) and stimulatory factor 2 (flow through of anion exchange column), and possibly the stimulatory factor from the heptyl-Sepharose column flow-through, from yPo1 II. We cannot eliminate the possibility that some of the polypeptides in yPo1 II* are stimulatory factors, but the absence of these polypeptides in the yPo1 II fraction makes this possibility unlikely. The inability of Budd et al. (9) to assay yPo1 II in the absence of the stimulatory factors may be due to the loss of enzymatic activity at low protein concentrations, a phenomenon noted by them and also observed by us.

Based on the following data, we propose that yPo1 II* is a possible holoenzyme form of yPo1 II. (i) The 145-kDa polypeptide of yPo1 II and the 200-kDa polypeptide of yPo1 II* are the catalytic subunits (Fig. 3). (ii) The antiserum to yPo1 II* specifically recognizes yPo1 II but not yPo1 I or yPo1 III (Fig. 4). (iii) Peptide mapping of the catalytic subunits of yPo1 II and yPo1 II* using proteases of different specificities produces many co-migrating polypeptides, with significant matches of the antigenic proteolytic fragments (Fig. 5). (iv) The polypeptide associated with the 200-kDa catalytic subunit of yPo1 II* co-elute over five columns and co-sediment in glycerol gradients (Fig. 6) with the polymerase activity with no change in their relative stoichiometries. (v) The molecular mass of 300 kDa for yPo1 II*, calculated from the sedimentation coefficient and the Stokes radius (Table II), is larger than the size of the catalytic subunit alone suggesting an association with the other subunits.

Although yPo1 II and yPo1 II* can be physically distinguished by their immunological reactivity and their polypeptide compositions, biochemically they are very similar. The processivities of both forms of yPo1 II are high with yPo1 II* having a somewhat higher processivity than yPo1 II (Fig. 9). Both yPo1 II and yPo1 II* appear to be capable of interacting

### Table IV

<table>
<thead>
<tr>
<th>Activity of DNA polymerases on various templates</th>
<th>yPol I/primase</th>
<th>yPol III</th>
<th>yPol II</th>
<th>yPol II*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated calf thymus DNA</td>
<td>219.0 (100)</td>
<td>45.0 (100)</td>
<td>176.0 (100)</td>
<td>243.0 (100)</td>
</tr>
<tr>
<td>Poly(dA):d(T)</td>
<td>90.2 (41)</td>
<td>3.3 (13)</td>
<td>87.4 (90)</td>
<td>335.0 (137)</td>
</tr>
<tr>
<td>Poly(dT):d(A)</td>
<td>6.2 (2.8)</td>
<td>0.6 (1.3)</td>
<td>0.5 (0.3)</td>
<td>0.8 (0.3)</td>
</tr>
<tr>
<td>Poly(dG):d(T)</td>
<td>55.2 (25)</td>
<td>1.2 (2.7)</td>
<td>7.8 (4.4)</td>
<td>21.6 (8.9)</td>
</tr>
<tr>
<td>Poly(dT):d(A)</td>
<td>30.0 (13)</td>
<td>1.5 (3.3)</td>
<td>0.7 (0.4)</td>
<td>0.2 (0.1)</td>
</tr>
<tr>
<td>Poly(dT) + ATP + Klenow</td>
<td>10.2 (4.7)</td>
<td>2.6 (5.8)</td>
<td>5.2 (3.0)</td>
<td>9.2 (3.8)</td>
</tr>
</tbody>
</table>

* Values in parentheses are percentages.

**FIG. 8. Inhibition of yeast DNA polymerases by aphidicolin and BuPdGTP.** Standard polymerase assays using activated calf thymus DNA as template were performed in the presence of the indicated amounts of aphidicolin (A) or BuPdGTP (B) except for the following changes: the concentration of dCTP was lowered to 10 μm in A, dGTP was lowered to 10 μm in B, and KCl was added to 100 mM for yPol II and yPol II*. ○—○, 1 unit of fraction V-C yPol I; ●—●, 0.2 unit of fraction VII-A yPol III; □—□, 0.3 unit of fraction VIII-D yPol II; ■—■, 0.3 unit of fraction VIII-E yPol II*.
with calf thymus PCNA in a manner that increases their already high processivities (Fig. 9). Some differences, however, were observed. yPol II* is stimulated by a slightly higher KCl concentration, has a broader Mg$^{2+}$ optimum, and is more active on poly(dA):oligo(dT) than yPol II. Further work will be required to clarify the biochemical differences between yPol II and yPol II* and to elucidate the possible functions of the yPol II* subunits.

Although it is difficult to distinguish yPol II from yPol II* by their biochemical properties, both forms of yPol II are readily distinguished from the other yeast DNA polymerases. The similarity of yPol II and yPol II* to the δ DNA polymerases is indicated by their BuPdGTP resistance (Fig. 8) and associated exonuclease activity and thus distinguishes them from yPol I, which is similar to the α DNA polymerases. yPol III is also similar to the δ DNA polymerases (7) but may be differentiated from yPol II by the sensitivity of yPol III to KCl (data not shown) and the stimulation of yPol II by KCl (Fig. 7). A major difference between the two δ-like DNA polymerases from yeast is that yPol III has a low processivity on poly(dA):oligo(dT) (38) whereas yPol II and yPol II* have a high processivity on this template:primer (Fig. 9). The processivities of both yPol II and yPol III is increased by calf thymus PCNA but this stimulation is much higher for yPol III. yPol III is therefore more similar to the δ DNA polymerase...
described by Downey et al. (36) that has low processivity but is greatly stimulated by PCNA.

If the similarities seen between yPol I and pol α (structural conservation of the polymerase-primase complex) and between yPol III and pol δ (ability of the heterologous calf thymus PCNA to stimulate yPol III) suggest a remarkable conservation of polymerase species between disparate organisms, then it may be possible to link yPol II to its higher eukaryotic counterpart. Several similarities can be noted between yPol II* and higher eukaryotic polymerases. The efficient utilization of poly(dA):oligo(dT) by yPol II* and the insensitivity to stimulation by PCNA are similar to the pool δ of Crute et al. (34), although the exonuclease activity of calf thymus pol δ II is inhibited by 5 mM AMP while that of yPol II* is not. A more striking similarity is observed between yPol II* and the human cell pol δ of Syvaoja and Linn (33). Both enzymes are not stimulated by PCNA, are highly processive on poly(dA):oligo(dT), are stimulated on activated calf thymus DNA templates by KCl, and are highly asymmetric molecules. The human pol δ has a 215-kDa polypeptide that could correspond to the 200-kDa subunit of yPol II*. A more thorough analysis will be required to establish homology between these polymerases.

The exonuclease activity associated with yPol II and yPol II* most likely resides in the polymerase catalytic subunit rather than the other subunits of yPol II* since yPol II lacks these subunits but still has exonuclease activity. The direction of excision is the 3′-5′ direction (Fig. 10). We are currently exploring the ability of the associated exonuclease activity to function in proofreading by the use of a system to measure the fidelity of DNA synthesis (41).

DNA polymerases, because they have a catalytic rather than a structural function, are expected to be of low abundance in the cell. We have found that the processing of large quantities of cells, especially for yPol II, was necessary in order to carry the purification through to homogeneity. Based upon the recovery of polymerase activity and assuming that yPol III is 50% pure, we estimate that the Mono Q pools having the resolved DNA polymerase species contain 475 μg of yPol I-primase complex, 175 μg of yPol II, 491 μg of yPol III, and 900 μg of yPol II* for every kilogram of yeast cells processed. Although estimates of protein amount based upon recovery of activity are subject to error if accessory or stimulatory factors are separated from the DNA polymerase, they indicate that to a first approximation none of the DNA polymerase species are dramatically over- or under-expressed in yeast. Although the principal form of yPol II appears to be yPol II*, a significant proportion has been degraded to the 145-kDa yPol II. We do not know whether this has occurred within the cells or during the early stages of purification because the antibody to yPol II* cannot detect y Pol II* in crude protein fractions (not shown). A similar phenomenon is observed with yPol I: we are able to purify yPol I as a proteolyzed 140-kDa catalytic subunit associated with the 70-kDa subunit but with the 58- and 48-kDa primase subunits absent (data not shown). The significance of this proteolysis is unknown, but it may indicate a regulatory role in the ability of the polymerases to form complexes with other subunits.

The presence of multiple DNA polymerase species in yeast raises the obvious question of what aspect of DNA metabolism is performed by each DNA polymerase. Yeast DNA polymerases I and III have already been shown to be required for DNA replication (14–17). The cellular function performed by yPol II remains to be determined. The similarity of yPol II* to the HeLa cell pol δ, which has been implicated in DNA repair (42), suggests that yPol II may function as a repair polymerase. With the isolation and identification of the major DNA polymerase species in yeast now complete, we are presently attempting to clone the genes for the yPol II* polymerases. The genetic approach available in the yeast system will be necessary to determine the in vivo function of yPol II and its possible subunits and may be relevant to those systems in which a genetic approach is not possible.

Acknowledgments—We thank Dr. L. Chang for the yeast DNA polymerase I immunoaffinity column, Dr. P. Burgers for providing antiseraum against yPol III, Dr. C.-K. Tan for generously providing calf thymus PCNA, and Dr. A. Morrison and Dr. D. Thomas for their critical reviews of the manuscript.

REFERENCES

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Nucleic Acids. Nucleic acids used during the course of purification and to quantitate nuclear activity were measured by the addition of acid-soluble radioactive (3H-thymidylate). The reaction mixture (25 ml) contained 1 ng/ml of T4 holoenzyme, 10 mM MgCl2, 200 mM Tris-Cl, pH 8.0, 100 mM NaCl, and 10 mM dithiothreitol at a constant temperature of 20°C. At the indicated time point, the sample was centrifuged and the supernatant was added to the reaction mixture and the reaction was stopped by an added volume of 4 ml at 4°C, and 20 µl of the supernatant was injected into Eollelum fluxid solution. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the incorporation of 1 pmole of thymidylate into DNA per minute in a reaction mixture.

Terminal mismatch excision was assayed on a mismatched substrate constructed by hybridizing a 15-base-paired substrate (180 bp) to a 27-base template oligonucleotide. This created a 3-nt (template) n gap at the 3'-terminus. The hybridization procedure oligonucleotide presented was shown to be a gap from the gap annealing.

Measurement of DNA polymerase activity on DNA of purified DNA polymerase. Polymerase activity was determined using dithiothreitol DNA template (10 ng/ml) for 30 s at 37°C in standard reaction conditions.

**Extraction of Protein:** Protein content was determined by measuring the absorbance at 280 nm of the extract using a spectrophotometer.

**Purification of DNA Polymerase II and I**

CB 104 yeast cells were grown at 30°C in YEPD (2%) medium containing 10 µg/ml of 3H-thymidylate to late log phase (10^9/ml cells). The cells were collected by centrifugation with a Shuler centrifuge and the cell paste was washed once with 500 ml of distilled water. All buffers used in subsequent steps contained 5 mM EDTA, 1 mg/ml DNAse, 1 mg/ml RNase, 0.1 M NaCl, 10 mM MgCl2, 100 µM 3H-thymidylate, 100 µM thymidine, 100 µM cytosine, 100 µM guanine, and 100 µM uracil.

**DNA Polymerase Assays:** DNA polymerase activity was assayed by measuring incorporation of [3H]-thymidylate into trichloroacetic acid-insoluble material. When activated yeast DNA template was used as the template, the reaction mixture (20 µl) contained 50 mM Tris-Cl, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol, 100 µg/ml bovine serum albumin, 10 mM glycerol, 50 µM of each dNTP, and 0.5 ng/ml of DNA polymerase. The reaction was started by adding 2 µl of phage T4 DNA polymerase (1000 units/ml). The reaction was allowed to proceed for 20 min. The reaction was stopped by adding 0.2 ml of an ice-cold solution containing 5% polyethyleneglycol and 50 mM NaCl, and centrifuged. The supernatant was subjected to electrophoresis in a 5% polyacrylamide gel for 48 h at 200 V to separate DNA. The DNA was stained with ethidium bromide, and the DNA was eluted from the gel.
Yeast DNA Polymerase II

VII-D. Phospho-Sepharose Chromatography. An equal volume of 2 M ammonium sulfate in Buffer T was added to Phospho-Sepharose and the sample was applied to a Phospho-Sepharose HR 5/5 column equilibrated with 1 M ammonium sulfate in Buffer T. The column was washed with 5 ml of 1 M ammonium sulfate in Buffer T and the protein eluted with a 30 ml linear gradient of 1 M ammonium sulfate in Buffer T. Fractions containing polymerase activity, eluting at 240 mM NaCl, were pooled and dialyzed overnight against 50 mM Tris-HCl, pH 8, 30% glycerol, 1 mM EDTA, 150 mM NaCl, and 15 mM 2-mercaptoethanol. Dithiothreitol (15 mM final concentration) was added to the dialyzed sample and the sample stored at -20°C (Fraction VIII-D).

VIII. Purification of RNA Polymerase III from Mouse E. coli

VIII-E. RNA Polymerase III. A crude extract of mouse E. coli was prepared as described above and centrifuged at 100,000 g for 1 hour. The superantigen was applied to a DEAE-cellulose column equilibrated with 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 50 mM 2-mercaptoethanol, and 30% glycerol. Fractions containing polymerase activity were pooled (Fraction VIII-E).

VIII-F. Phenyl-Sepharose Chromatography. An equal volume of 2 M ammonium sulfate in Buffer T was added to Fraction VIII-E and the sample was applied to a Phenyl-Sepharose HR 5/5 column equilibrated with 1 M ammonium sulfate in Buffer T. The column was washed with 5 ml of 1 M ammonium sulfate in Buffer T and the protein eluted with a 30 ml linear gradient of 1 M ammonium sulfate in Buffer T. Fractions containing polymerase activity, eluting at 300 mM ammonium sulfate, were pooled and dialyzed against Buffer T containing 10% Tris-X-100. The dialyzed sample was stored at -20°C (Fraction VIII-F).

VIII-G. Single-stranded DNA Cellulose Chromatography. Fraction VIII-E was applied to a single-stranded DNA cellulose column in an HR 5/5 column and equilibrated with 50 mM NaCl in Buffer T containing 10% Tris-X-100. The column was washed with 5 ml of Buffer T and the protein eluted with a 30 ml linear gradient from 50-100 mM NaCl in Buffer T containing 10% Tris-X-100. Fractions containing polymerase activity, eluting at 240 mM NaCl, were pooled and dialyzed overnight against 50 mM Tris-HCl, pH 8, 30% glycerol, 1 mM EDTA, 150 mM NaCl, and 15 mM 2-mercaptoethanol. Dithiothreitol (15 mM final concentration) was added to the dialyzed sample and the sample stored at -20°C (Fraction VIII-G).

VIII-H. Purification of DNA polymerase II from E. coli

IX. Purification of DNA polymerase II from E. coli

IX-A. Phenyl-Sepharose Chromatography. Phenyl-Sepharose (2 ml) was applied to a Phenyl-Sepharose HR 5/5 column equilibrated with 50 mM NaCl in Buffer T. Fractions containing polymerase activity, eluting at 300 mM sodium chloride, were pooled and dialyzed overnight against 50 mM Tris-HCl, pH 8, 30% glycerol, 1 mM EDTA, 150 mM NaCl, and 15 mM 2-mercaptoethanol. Dithiothreitol (15 mM final concentration) was added to the dialyzed sample and the sample stored at -20°C (Fraction IX-A).
Purification and characterization of DNA polymerase II from the yeast Saccharomyces cerevisiae. Identification of the catalytic core and a possible holoenzyme form of the enzyme.
R K Hamatake, H Hasegawa, A B Clark, K Bebenek, T A Kunkel and A Sugino